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SOME ENDOCRINE FACTORS IN THE POLYURIC ACTION OF RENIN[1]

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DIFFERENT authors have shown that the injection of renin produces polyuria in rabbits (Pickering, 1940; Brandt *et al.*, 1948; Hughes, *et al.*, 1949); and in rats (Addis *et al.*, 1949; Masson *et al.*, 1950; Sellers *et al.*, 1951; Croxatto *et al.*, 1951).

The polyuric effect of renin is abolished by adrenalectomy in rats maintained on 1% NaCl drinking solution (Croxatto *et al.*, 1952).

The different behavior of renin in normal and adrenalectomized rats is observed both in animals normally hydrated with free intake of water and in water-loaded animals. Whether the adrenal factor involved in the action of renin arose from the cortex or medulla and whether or not it involved interaction with either the anterior or posterior pituitaries are questions left unanswered by past work. The present experiments were designed to provide such answers.

MATERIALS AND METHODS

Pig renin was prepared according to the method of Braun Menendez (1943). The renin preparation contained 15 to 25 U per ml. and was free of hypertensinase. Sodium chloride was added to provide isotonicity. The adrenal steroids used were: Cortisone (Merck), 4 mg. per ml. in aqueous suspension; desoxycorticosterone glucoside (DCG-Syntex), dissolved in distilled water at 10 mg. per ml. and desoxycorticosterone acetate (DCA-Schering), 10 mg. per ml. in oil solution.

Adult male and female rats of an average weight of 180 to 240 gm. were used in metabolic cages. In this study, as in a previous one (Croxatto, 1952), both normally hydrated and water-loaded animals were used.

Received for publication June 28, 1953.

[1] We thank the Gildemeister Foundation and the Rockefeller Foundation for the assistance given to this work.

Animals on normal hydration. These rats had free access to water. Hypophysectomized animals were kept in individual cages; in other cases, four rats were kept per cage. The injections were made at 8 A.M. and the urine was measured during the following twelve hours. During this period, the rats, although on water *ad libitum*, were deprived of food. The water intake and the urine volume are expressed as ml. per 100 gm. body weight per 12 hours.

Series A comprised 32 adrenalectomized animals, distributed in four groups of eight rats each and the same number of groups of normal animals previously subjected to an observation period during which the diuresis and NaCl consumption were controlled.

Of the four groups of adrenalectomized animals, the first was injected with 2 mg. of DCG; the second with 0.8 to 2 mg. cortisone diluted in 0.2 ml; the third with 2 mg. DCA in 0.2 ml. oil; and the fourth with the same volume of 0.9% NaCl daily. The normal animals were distributed and injected in the same way, receiving the same doses of hormones or 0.9% NaCl. The hormones were administered during the whole experimental period.

On the sixth day of hormone administration, an intraperitoneal injection of 1 ml. (15 to 25 U) of renin daily was started in all the animals. After 6 days the renin injections were discontinued, but the injections of hormones were still given and daily water exchange recorded. Immediately afterwards the injections of renin were repeated, subcutaneously this time.

In a second phase of the experiment, the 1% NaCl solution was replaced by plain water in all the groups of animals, except the fourth. After 5 days, the injections of renin were continued, first intraperitoneally for 5 days, followed afterwards by subcutaneous injections.

In Series B the medullary portion of the adrenals was removed by "enucleation" in four rats. Three weeks after the operation, they were injected with 1 ml. renin intraperitoneally, using as controls the same number of normal animals. As in the animals of Series A, they received 1% NaCl solution at a first stage and plain water afterwards as a drinking fluid.

In Series C the hypophysis was completely removed (through the pharynx) from a large number of rats. From these, those having definite and permanent polyuria were selected and placed in cages 5 to 60 days after hypophysectomy. Twenty of these rats were injected with 1 ml. of renin intraperitoneally every 12 to 24 hours, ten of them being given water to drink and the other ten being given 1% NaCl. 0.9% NaCl was injected intraperitoneally instead of renin to ten other hypophysectomized animals used as a control group.

All hypophysectomized animals were killed at the end of the experiment to investigate the presence of rests of hypophysis. In the pieces that were suspicious, the antidiuretic properties were assayed to identify the presence of vasopressin. The hypophysectomy was considered total when diabetes insipidus developed and no rests of hypophysis were found.

Animals on Forced Hydration. Rats deprived of any food for 12 hours and placed in metabolic cages were injected with 1 ml. renin intraperitoneally. Two hours later, 5 ml. per 100 gm. of water were given through a gastric tube, after which urine excretion was measured every 15 minutes.

In Series E the animals were distributed in the same way as those of Series A., i.e., four groups of 8 adrenalectomized rats each. For 20 days previously they had received respectively, DCG, cortisone, DCA and 0.9% NaCl, in the doses for series A.

Series F included 12 recently hypophysectomized animals (5 to 7 days) and 12

animals operated 15 to 60 days previously. Half of the animals were injected with 1 ml. renin intraperitoneally and the other half with the same volume of 0.9% NaCl.

Series G consisted of 12 normal and 12 hypophysectomized animals that had received a daily injection of 1 mg. cortisone for 5 to 15 days. They were hydrated with plain water 2 hours after being injected with 1 ml. renin intraperitoneally. Controls consisted ot the number of animals, identically treated but minus the cortisone.

RESULTS

Series A: In Figures 1 and 2 the diuresis results obtained on adrenalectomized and normal animals are shown with and without injected hormones and before and after the administration of renin.

The most evident and constant phenomenon is the intense stimulation of diuresis by renin in all the normal animals and in those operated animals receiving DCA and cortisone. Two groups of operated animals injected with DCG are the exception; the polyuric action is either absent or very weak. It has also been established that adrenalectomized animals maintained exclusively on 1% NaCl are completely refractory to this polyuric action of renin.

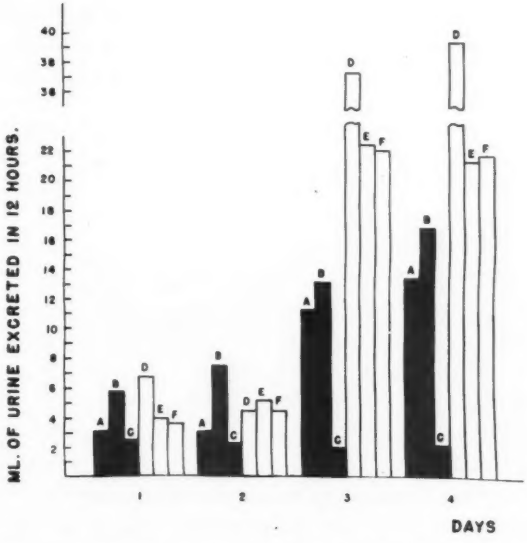

FIG. 1. Each bar expresses the average volume of urine, in ml., excreted per 100 gm. of rat in 12 hours. Animals given solution of 1% NaCl to drink. The black bars represent adrenalectomized animals, and the white bars normal ones. Four animals per group. Daily subcutaneous injection as follows: A and D. 2 mg. DCA in oil. 0.5 ml.; B and E. 0.8 mg. Cortisone in 0.5 ml. of 0.9% NaCl; C and F. 0.5 ml. NaCl (0.9%). During days 3 and 4 0.5 ml. of renin was injected daily per 100 gm. of rat *intraperitoneally.*

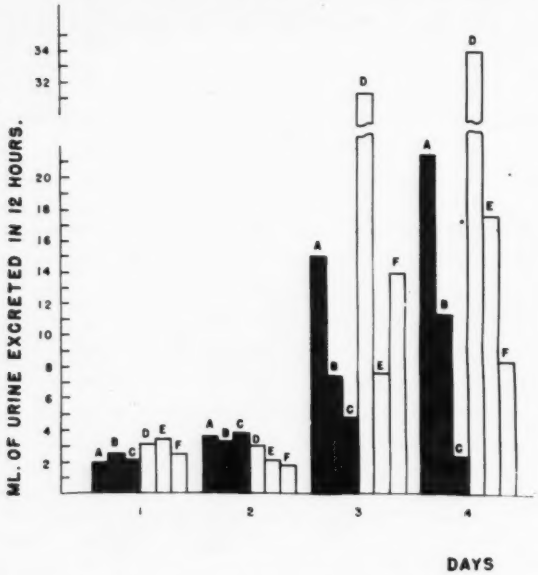

FIG. 2. Each bar expresses the average volume of urine in ml. excreted per 100 gm. of rat during 12 hours. Animals drinking a solution of 1% NaCl. The black bars represent adrenalectomized animals and the white bars normal animals. Four animals per group. Daily subcutaneously injections as follows: A and D. 2 mg. DCA in oil, 0.5 ml.; B and E. 0.8 mg. Cortisone in 0.5 ml. of 0.9% NaCl solution; C and F. 0.5 ml. 0.9% NaCl. During days 3 and 4 0.5 ml. of renin was injected daily per 100 gm. of rat *subcutaneously*.

Cortisone in doses of 0.8 to 2 mg. and DCA at a dose of 2 mg. are apparently sufficient to restore the diuretic response to renin in the adrenalectomized animal to that characteristic of normal animals. Nevertheless, the normal animals injected with DCA, which receive renin either intraperitoneally or subcutaneously, present a remarkably higher diuresis than that of similarly-treated adrenalectomized animals.

It was observed in some experiments that cortisone injections in adrenalectomized rats receiving plain drinking water induced a more intense polyuric response to renin than normal animals treated in the same way. The highest diuresis was registered in the group of normal rats receiving renin 1% NaCl and 2 mg. DCA daily. Each rat excreted an average of 60 ml. in 12 hours—an excretion of 42% of its weight daily.

In the group of operated animals injected with 0.9% NaCl, without hormone administration, the average excretion per rat in a 12-hour period was 4 ml. before renin injection and 3.8 during the injection of renin (Figs. 1 and 2).

In studying the effect of hormones on the diuresis of normal rats previous to renin injection, it was observed that a dose of 0.8 mg. of cortisone produced a slight increase in diuresis as compared to the normal group without hormone injection. Nevertheless, in a dosage of 2 mg./rat/day, cortisone increased the volume of excreted urine noticeably; it was two or three times higher than that of controls. This stimulating effect of cortisone on diuresis was also observed in the adrenalectomized animals, particularly after several injections. A similar phenomenon was observed with 2 mg. DCA in normal animals. Steroid-treated animals, however, never reached the level of diuresis observed in normal animals receiving renin alone.

Series B: Demedullated animals responded to renin and in other respects behaved like normal animals.

Series C: Hypophysectomized animals receiving drinking water did not respond to the injection of renin as normal control animals did. The excretion of urine was the same as that of animals which received 0.9% NaCl injections (3 A, B). The administration of 1% NaCl made these animals sensitive to renin; the polyuric action resulting from this drinking solution was less than that of normal animals (3 C).

Series D: The administration of cortisone in hypophysectomized animals produced a noticeable increase in the diuretic response to renin.

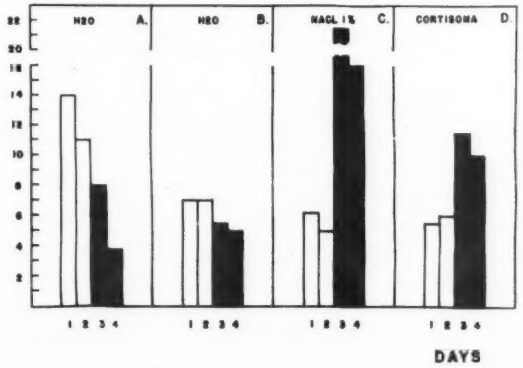

FIG. 3. Each bar represents the average volume of urine in ml. excreted in 12 hours per 100 gm. of rat by completely hypophysectomized animals with diabetes insipidus. Black bars represent the period when the following was injected intraperitoneally per 100 gm. body weight: A. 0.5 ml. of renin (water to drink). Group of 10 hypophysectomized rats; B. 0.5 ml. of 0.9% NaCl (water to drink). Group of 10 hypophysectomized rats; C. 0.5 ml. of renin (1% NaCl to drink). Group of 10 hypophysectomized rats; D. 0.5 ml. of renin, after 8 days of treatment with 0.8 mg. Cortisone daily (water to drink). Group of 6 hypophysectomized rats.

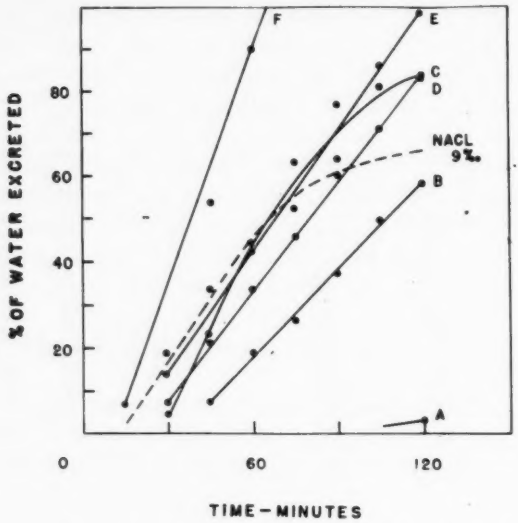

FIG. 4. Percentage of water load excreted in 7 groups of 4 rats after the administration of water through a gastric tube (5% of body weight, Burn's test). Animals on plain water to drink: Groups A, B and C. adrenalectomized; Groups D, E and F. normal. Previous daily treatment (20 days): Groups A and D. 0.5 ml. 0.9% NaCl subcutaneously; Groups B and E. 2 mg. DCA; Groups C and F. 2 mg. Cortisone. Two hours prior to hydration, all the animals were injected with 0.5 ml. renin per 100 gm. of body weight intraperitoneally. The dotted line corresponds to the urinary excretion of a control group of normal rats injected with 0.9% NaCl instead of renin.

Again, the response was less than that of normal animals. This result is comparable to that observed in adrenalectomized rats treated with the hormone (3 D).

Even before receiving cortisone, hypophysectomized rats showing the presence of anterior pituitary remnants responded to renin with a slight increase in diuresis. In this group cortisone produced a definite increase in the volume of urine during the 5-day injection period. Despite the fact that the hypophysectomized rats present a higher than normal basal diuresis, evidently cortisone and renin do not produce in these animals the very intense diuretic effect observed in normal and adrenalectomized animals treated with hormones.

Series E: The experiments performed in this group prove that adrenalectomy prevents the accelerated urinary excretion in those animals subjected to forced hydration and given renin. Adrenalectomized rats given 1% NaCl do not respond to renin; in fact, the urinary excretion was retarded. Even after hydration, a prolonged phase of anuria sometimes appeared (water intoxication).

The daily administration of 2 mg. DCA or 0.8 mg. cortisone not only reestablished the capacity of the rats to respond to renin as in unoperated animals, but the resulting polyuria reached the high levels obtained in normal animals given renin 2 hours before forced hydration and treated with the same dose of cortisone or DCA (Figs. 4 and 5). DCG did not modify the response of adrenalectomized animals.

Series F: Total hypophysectomy banished completely, or almost completely, the capacity for responding to renin with accelerated urinary excretion. The diuresis in these animals was identical, or slightly higher, than that observed when 0.9% NaCl was injected at equivalent volume in place of renin (Fig. 6). In recently hypophysectomized rats (5 days), with adrenal cortical hypofunction not as marked, this result was also true.

Series G: Previous cortisone administration to hypophysectomized, water-loaded animals increased the speed of water excretion after renin injection, reestablishing this phenomenon almost to the levels seen in intact animals given similar treatment.

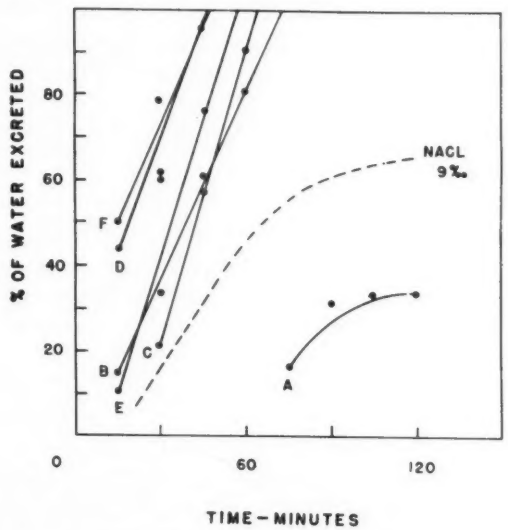

FIG. 5. Percentage of water load excreted in 7 groups of 4 rats after the administration of water through a gastric tube (5% of body weight, Burn's test). Animals on 1% NaCl drinking solution: Groups A, B and C. adrenalectomized; Groups D, E and F. normal. Previous daily treatment (20 days): Groups A and D. 0.5 ml. 0.9% NaCl subcutaneously; Groups B and E. 2 mg. DCA in oil subcutaneously, Groups C and F. 0.8 mg. Cortisone subcutaneously. Two hours prior to hydration, all the animals were injected with 0.5 ml. of renin per 100 gm. of body weight intraperitoneally. The dotted line corresponds to the urinary excretion of a control group of normal rats injected with 0.9% NaCl instead of renin.

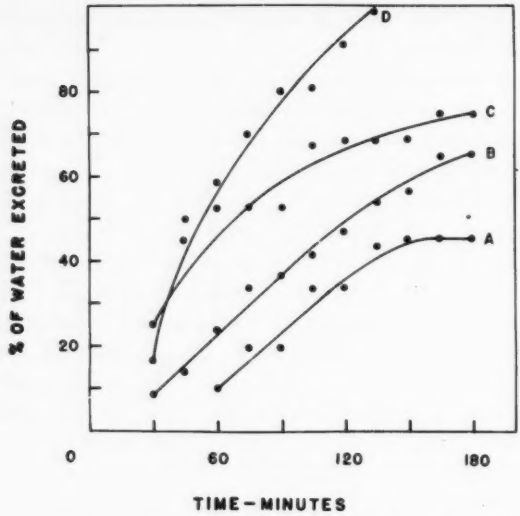

FIG. 6. Percentage of water load excreted in 4 groups of 4 hypophysectomized rats after the administration of water through a gastric tube (5% of body weight. Burn's test). Two hours prior the hydration, there was injected intraperitoneally and per 100 gm. of body weight, in: A. 0.5 ml. of 0.9% NaCl; B. 0.5 ml. of renin; C. 0.5 ml. of 0.9% D. 0.5 ml. of renin. The rats of groups C and D were previously treated for 8 days with 0.8 mg. of cortisone daily.

DISCUSSION

The experimental data confirms the information described by Croxatto *et al.* (1952), i.e., that adrenalectomy enables normally hydrated animals and those on forced hydration to respond with a rise of diuresis after the injection of renin.

This result is not surprising since Rowntree and Snell (1931), in Addisonian patients, and Silvette and Britton (1933), in adrenalectomized cats, showed that water was excreted at a markedly delayed rate. Gaunt *et al.* (1937) in both adrenalectomized and hypophysectomized rats, noted a great susceptibility to water intoxication, an essential fact abundantly confirmed by many investigators. When renin is injected, the inability of rats to excrete water at a higher rate could be added as another sign to the other symptoms of adrenal insufficiency.

The incapacity of the adrenalectomized animal is entirely, or almost entirely, corrected with DCA or cortisone. This indicates that these and similar-acting hormones produced by the adrenal cortex are indispensable to the polyuric action of renin.

The observation that demedullated animals respond to renin normally

confirms the paramount role of the adrenal cortex. This excludes the possibility that adrenalin and noradrenalin play a part in the polyuric effect produced by renin.

DCG ineffectiveness could be due to the dose administered or to a poor absorption when administered subcutaneously. Frequently, persistent indurations appeared at injection sites after DCG injections, a response that did not occur after the injection of DCA in oil.

Although the adrenal cortical hormones affect the polyuric action of renin, there is no reason to believe that renin causes a stimulation of the adrenal cortex. It is still questionable whether the action of cortical hormones results from the inhibition of the reabsorption of water in the kidney or from its influence on the hydro-saline metabolism of the extracellular space and tissues.

It seems probable that the saline concentration of the body fluids plays the most important role. The degree of ingestion of NaCl in the normal animal has a definite affect on the magnitude of the polyuric effect of renin.

The effects of total hypophysectomy are in agreement with those of adrenalectomy. Renin does not increase the diuresis of the hypophysectomized animal—in spite of a state of diabetes insipidus.

Lack of response of the hypophysectomized animal is probably due largely to hypofunction of the adrenal cortex. This is suggested by the fact that cortical hormones partially relieve the deficiency in the diuretic response to renin. Other factors, however, must also be involved since cortisone did not relieve the deficiencies of the hypophysectomized animals completely nor as well as it did in adrenalectomized rats.

The polyuric action of renin is not produced, of course, by mere inhibition of the endocrine activity of the neurohypophysis, i.e., renin polyuria is not equivalent to a diabetes insipidus from temporary inhibition of secretion of vasopressin.

The sum total of all observations would indicate that the polyuric action of renin is manifest whenever the organism has normal quantities of chlorine and sodium available. When adrenal insufficiency is produced by adrenalectomy or hypophysectomy, subnormal body stores of those ions would make the diuretic effect impossible. An excess of NaCl raises the sensitivity of the animals to renin. Other factors should be studied, however, to explain completely the deficit in adrenalectomized and hypophysectomized animals.

SUMMARY AND CONCLUSIONS

The conditions which permit restoration of the diuretic response to renin were studied in adrenalectomized and hypophysectomized rats. In

addition, the effects of various endocrine manipulations were observed in intact rats. Conclusions were:

1) In adrenalectomized animals, the administration of 0.8 to 2 mg. of cortisone or 2 mg. of DCA per rat per day (adult animals of 200 to 250 gm.) restores the ability to respond to renin by a polyuria. This was seen in normally hydrated rats with free ingestion of liquids and in rats subjected to forced hydration.

2) The extirpation of the adrenal medulla was without effect on the response to renin.

3) The injection of renin did not produce diuretic effects in completely hypophysectomized animals (5 to 60 days) with diabetes insipidus. The administration of cortisone or DCA markedly restored the capacity for rapid water excretion when these animals were hyperhydrated. A similar effect followed treatment with 1% NaCl as a drinking fluid.

The polyuric action of renin is not due to a direct or indirect stimulation of the adrenal cortex nor can the phenomenon be attributed to a temporary inhibition of the neurohypophysis by decreasing antidiuretic hormone production. The adrenocortical hormones are, however, indispensable for the diuretic effect above mentioned. The essential and conditioning role of these hormones in the diuretic action of renin could be due to their action on the metabolism of Cl and Na, for the intensity of the diuretic effects of renin are markedly influenced by the ingestion of NaCl.

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IDENTIFICATION OF THE EFFECTS OF THYROXINE MEDIATED BY THE HYPOPHYSIS

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ACCORDING to the concepts of Classical Endocrinology, an *effect* of thyroxine may be defined as the restoration by this hormone[1] of a physiological process altered by thyroidectomy. Such effects are not necessarily due to a direct action of thyroxine on an end organ; some may result from complex hormonal interactions. Thus, Evans and his associates have suggested that the growth-promoting effect of thyroxine is exerted through the hypophysis (Scow *et al.*, 1949; Koneff *et al.*, 1949; and Becks *et al.*, 1950).

It was decided to examine systematically the relationship between the hypophysis and the production of the effects of thyroxine. To this end, hypophysectomized animals were treated with replacement doses of thyroxine and the results obtained were compared with those observed in thyroidectomized animals similarly treated. The conclusions derived from this comparison helped to clarify the role played by the hypophysis in the production of thyroxine effects.

METHODS

This work consisted of 4 experiments carried out in male albino rats (Table 1). Experiment A was a preliminary test designed to investigate the effects of a small dose of thyroxine in hypophysectomized animals. Experiment B—the main one of this series—was planned to compare the effects of various doses of thyroxine in thyroidectomized and hypophysectomized rats. Experiment C repeated part of Experiment B in much younger rats. In the above 3 experiments, the animals were under investigation for about 3 weeks. Experiment D consisted of small groups of hypophysectomized rats maintained with and without thyroxine for 5 weeks.

Received for publication July 2, 1953.

[1] The arguments leading to the conclusion that L-thyroxine is a thyroid hormone have been summarized elsewhere (Leblond, 1951). However, in addition to L-thyroxine, thyroid tissue and plasma contain small amounts of a substance previously referred to as compound No. 1 (Gross and Leblond, 1951). This substance was recently identified as L-triiodothyronine and is endowed with biological activity (Gross *et al.*, 1952). At the present time it is tentatively assumed that both L-thyroxine and L-triiodothyronine are thyroid hormones.

In the appropriate groups of animals, thyroidectomy was performed as described previously by Leblond and Eartly (1952), the operation involving the removal of the parathyroids as well as the thyroid. The animals to be hypophysectomized were operated on by the Charles River Breeding Laboratories of Boston a week later to insure that the thyroidectomized and hypophysectomized controls had approximately the same body weights at the end of the experiment.

The animals were fed a djet consisting of corn meal (70%), wheat gluten (16%), yeast (10%), (Viobin) liver (2%), calcium carbonate (1%) and sodium chloride (1%). This diet was sufficiently low in thyroxine and iodine to produce a pronounced thyroxine deficiency (Leblond and Eartly, 1952). The thyroidectomized animals were given calcium lactate (1% solution) instead of drinking water for one week post-operatively. The hypophysectomized animals were given a 10% dextrose solution as drinking water throughout the experimental period. The doses of thyroxine administered daily are detailed in Table 1. The measurements of heart rate and oxygen consumption were carried out according to the method described by Grad (1952).

At the termination of the experiments, the animals were sacrificed with chloroform; their organs were fixed in Orth fluid, weighed, and subsequently sectioned and stained with hematoxylin and eosin for histological study.

RESULTS

Experiment A: Effect of a 3-week treatment with thyroxine in young adult hypophysectomized male rats

Examination of the *body weights* (Fig. 1) showed that growth stopped in all hypophysectomized animals and that those treated with 6 μg. of

TABLE 1. EXPERIMENTAL GROUPS

	Duration of experiment (days)	Group	Number of animals	Initial body weight (gm.)
Experiment A	19	Hypophysectomized control	10	140
		Hypophysectomized +6 μg. thyroxine	9	141
		Intact Control	10	143
Experiment B*	20	Thyroidectomized control	10	130
		Thyroidectomized +3 μg. thyroxine	10	134
		Thyroidectomized +6 μg. thyroxine	10	137
		Thyroidectomized +12 μg. thyroxine	10	141
		Hypophysectomized control	15	145
		Hypophysectomized +3 μg. thyroxine	15	144
		Hypophysectomized +6 μg. thyroxine	15	144
		Hypophysectomized +12 μg. thyroxine	15	144
		Intact control	10	146
Experiment C	19	Thyroidectomized control	20	38
		Thyroidectomized +4 μg. thyroxine	20	39
		Hypophysectomized control	20	45
		Hypophysectomized +4 μg. thyroxine	20	47
		Intact control	10	39
Experiment D	36	Hypophysectomized control	7	124
		Hypophysectomized +6 μg. thyroxine	7	127

* All thyroidectomized animals in this experiment were also castrated.

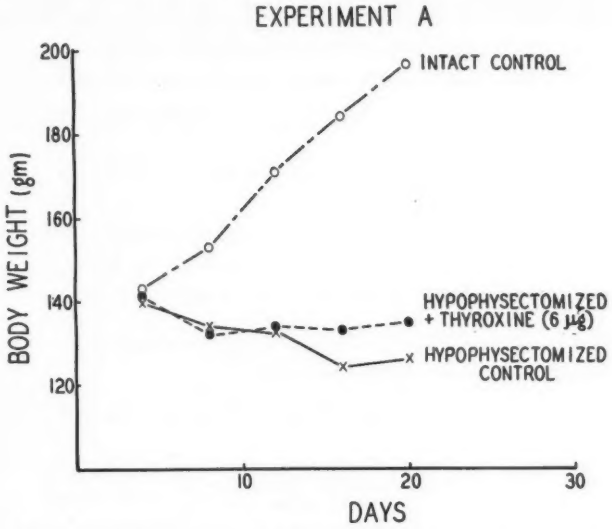

FIG. 1. Influence of thyroxine on the body weight of hypophysectomized male rats. Hypophysectomies were done on Day 0 and thyroxine started on Day 4.

The growth observed in the intact controls was absent after hypophysectomy, whether or not thyroxine was administered.

thyroxine daily did not gain significantly more than those which did not receive the hormone.

The *oxygen consumption and heart rate* (Table 2) were significantly raised by thyroxine and, at least in the case of the heart rate, restored to the same level as in the intact controls.

In regard to the *size of non-endocrine organs* (Table 3) the *absolute*

TABLE 2. INFLUENCE OF THYROXINE ON THE OXYGEN CONSUMPTION AND HEART RATE OF MALE HYPOPHYSECTOMIZED RATS
(Experiment A)

Days after operation	Hypophysectomized control	Hypophysectomized +6 μg. thyroxine	Intact control
	Oxygen consumption (cc./hr./100 sq.cm.)		
11	71.0	79.7	91.8
15	64.6	70.4	94.6
18	61.7	64.4	85.1
Mean	65.7	71.5	90.5
	Heart rate (beats per minute)		
11	277	304	307
15	307	325	333
18	305	359	376
Mean	295	329	338

weights were higher in thyroxine-treated than in control hypophysectomized animals, but in both cases they were much smaller than in the intact controls. When the weights were corrected for a body weight of 100 gm. $\left(\frac{\text{organ weight} \times 100}{\text{body weight}}\right)$, the *relative* organ weights thus calculated were found to be raised by thyroxine treatment to a level similar to that of the intact controls.

The *endocrine organs* (adrenals, thyroids, and testes) were atrophic in the hypophysectomized controls and were repaired in no way by thyroxine. In fact, this hormone further decreased the size of the testes (Table 3).

Experiment B: Comparison of the effects of thyroxine in young adult thyroidectomized and hypophysectomized male rats

Examination of the *body weights* (Fig. 2) showed that untreated thyroid-

TABLE 3. INFLUENCE OF THYROXINE ON THE ORGAN WEIGHTS OF HYPOPHYSECTOMIZED MALE RATS
(Experiment A)

	Hypophysectomized control	Hypophysectomized +6μg. thyroxine	Intact control
Final number of animals	6	7	10
Initial body weight (gm.)	140	141	143
Final body weight (gm.)	123	135	197
Organ weights			
Absolute (mg.)			
Relative (mg./100 gm. body weight)			
Kidney	867	1095	1690
	688	*810*	*856*
Heart	388	486	672
	307	*358*	*341*
Thymus	201	313	367
	158	*215*	*186*
Spleen	649	728	1052
	477	*541*	*540*
Liver	5120	5725	8594
	4049	*4242*	*4346*
Salivary glands	269	363	500
	214	*264*	*252*
Adrenals	12	12	30
	9.5	*9.0*	*15.0*
Testes	577	497	2617
	454	*346*	*1341*
Seminal vesicles	51	43	445
	38	*31*	*224*
Thyroid	12	12	23
	9.8	*8.8*	*12.0*

Any underlined figure is significantly different from that of the operated control group on the left of the same line.

ectomized animals continued growing, though at a reduced rate, for 10 days after operation and then plateaued, while hypophysectomized animals stopped growing. Doses of 3 or 6 μg. of thyroxine restored full growth (Fig. 2), while 12 μg. restored growth only partially in thyroidectomized

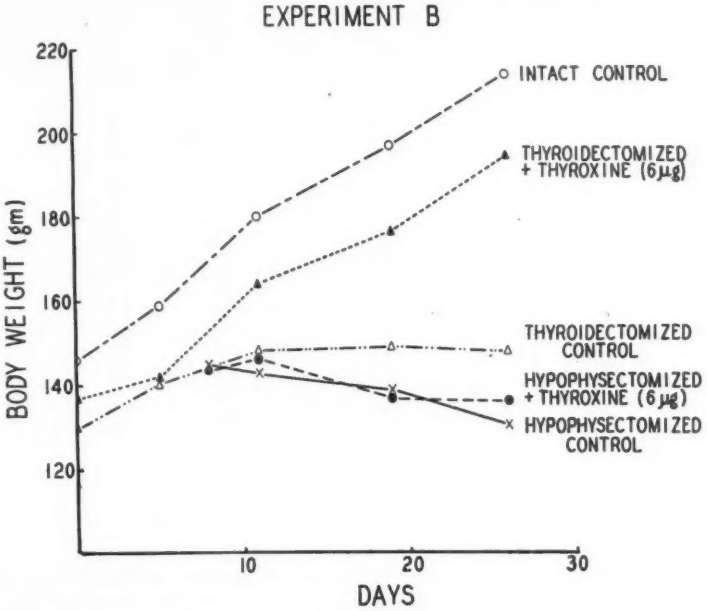

FIG. 2. Influence of thyroxine on the *body weight* of thyroidectomized and hypophysectomized male rats. On Day 0, thyroidectomies were carried out and thyroxine started in some of the operated animals. On Day 6, hypophysectomies were carried out, while on Day 8, thyroxine was started in some of the hypophysectomized animals. (For the sake of clarity, the action of thyroxine is illustrated by showing only the effect of a 6 μg. dose.)

Thyroidectomized animals continued to gain weight, although at a reduced rate, for approximately 10 days after operation, whereas hypophysectomized animals stopped growing (and even started to lose weight) immediately after operation. Furthermore, thyroxine restored the growth curve of thyroidectomized rats to normal, but was ineffective in hypophysectomized rats.

animals. (The latter dose probably was toxic for it raised the oxygen consumption above the normal level.) Thyroxine in any of these doses had no significant effect on the growth of hypophysectomized animals (Fig. 2).

The *oxygen consumption* (Fig. 3) was low in untreated thyroidectomized and even lower in untreated hypophysectomized rats, but was raised by administration of thyroxine in both types of animals. Graded doses of thyroxine evoked graded responses, which differed significantly between

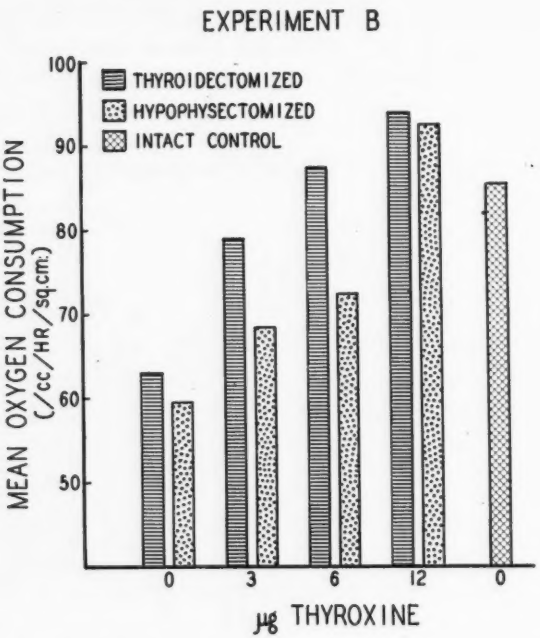

FIG. 3. Influence of various doses of thyroxine on the *oxygen consumption* of thyroidectomized and hypophysectomized male rats.

Graded doses of thyroxine produced proportionate increases in oxygen consumption in both types of animals. The oxygen consumption was significantly lower in hypophysectomized than in thyroidectomized animals, except with the 12 μg. dose of thyroxine.

groups. The level reached was higher in thyroidectomized than in hypophysectomized rats with 3 or 6 μg., but was about the same with 12 μg. of thyroxine.

The *heart rate* results (Fig. 4) were similar to the oxygen consumption findings. Graded doses of thyroxine produced a corresponding increase in heart rate. There was no significant difference between the responses of thyroidectomized and hypophysectomized animals.

The weights of *non-endocrine organs* (Table 4) tended to be restored to normal by thyroxine in thyroidectomized animals, while in hypophysectomized animals under the same treatment, only the relative weights approached normal. There was some indication of graded increases in the weights of kidney, heart, etc., in response to the graded doses of thyroxine (Table 4).

Thyroxine increased the size of the adrenal glands in thyroidectomized but not in hypophysectomized animals.

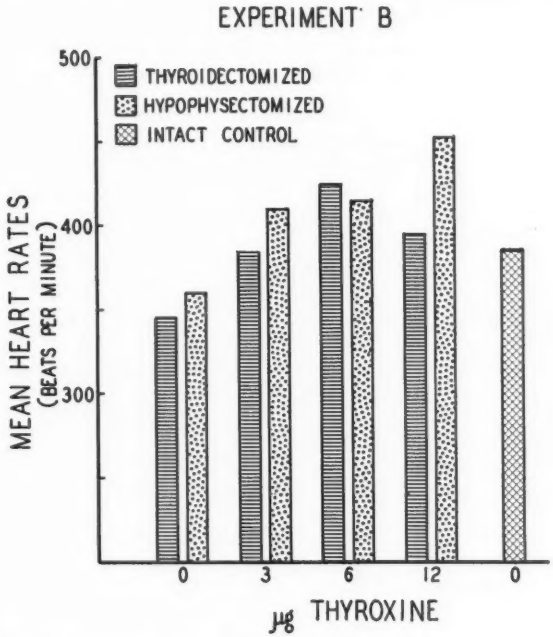

FIG. 4. Influence of various doses of thyroxine on the *heart rate* of thyroidectomized and hypophysectomized male rats.

Thyroxine increased the heart rate in both types of animals. The 3 and 6 μg. doses had approximately the same effect in thyroidectomized and hypophysectomized animals.

Histological studies revealed significant changes in the *kidney*. The diameter of the proximal convoluted tubules was decreased in thyroidectomized (Fig. 5) and even more in hypophysectomized (Fig. 7) rats. Thyroxine restored tubule diameter completely in the former (compare Figs. 6 and 9), but only to a very small extent in the latter (Fig. 8). The *submaxillary gland* of untreated thyroidectomized (Fig. 10) and hypophysectomized rats (Fig. 12) showed a reduction in the size and number of serous tubules, which were completely devoid of the acidophilic serous granules present in the intact controls (Fig. 14). Thyroxine treatment restored to some extent the size and granule content of the serous tubules in thyroidectomized (Fig. 11), but not in hypophysectomized animals (Fig. 13).

The *epidermis* was thicker in thyroidectomized and hypophysectomized animals than in intact controls. Treatment with thyroxine tended to decrease this thickness.

Examination of the Susa-fixed *hypophyses* of untreated thyroidectomized rats showed, as usual, a complete lack of acidophils, which defect was

TABLE 4. INFLUENCE OF THYROXINE ON THE ORGAN WEIGHTS OF THYROIDECTOMIZED AND HYPOPHYSECTOMIZED MALE RATS
(Experiment B)

	Operated control		3 μg. Thyroxine		6 μg. Thyroxine		12 μg. Thyroxine		Intact Control
	T-ect.	Hy-ect.	T-ect.	Hy-ect.	T-ect.	Hy-ect.	T-ect.	Hy-ect.	
Final number of animals	5	11	6	9	5	7	6	11	8
Initial body weight (gm.)	130	145	134	144	137	144	141	144	146
Final body weight (gm.)	148	130	208	139	194	136	174	130	214
Organ weight									
Absolute (mg.)									
Relative (mg./100 gm. body weight)									
Kidneys	958	938	1490	1110	1435	1098	1648	1172	1676
	650	*714*	*713*	*799*	*751*	*808*	*949*	*909*	*791*
Heart	566	436	748	558	715	578	764	569	771
	384	*332*	*358*	*400*	*376*	*425*	*442*	*436*	*364*
Thymus	279	250	400	334	347	352	345	284	362
	186	*192*	*191*	*240*	*185*	*268*	*198*	218	*168*
Spleen	652	625	1078	657	974	644	1120	689	1109
	440	*478*	*577*	*470*	*520*	*476*	*643*	*520*	*517*
Liver	5006	4910	8074	5206	7115	5526	7753	5652	8027
	3390	*3770*	*3840*	*3730*	*3730*	*3960*	*4470*	*4340*	*3730*
Salivary glands	361	288	520	340	486	324	583	286	521
	251	*222*	*249*	*244*	*252*	*239*	*335*	*219*	*243*
Adrenals	28	15	34	17	43	14	51	14	28
	20	*11*	*16*	*12*	*23*	*10*	*29*	*11*	*13*
Testes		541		479		457		396	2604
		415		*346*		*322*		*264*	*1212*
Thyroid		13		13		9		12	30
		10		*9*		*6*		*8*	*19*

Any underlined figure is significantly different from that of the operated control group on the left of the same line.

corrected by thyroxine treatment. The atrophied *testes* of the hypophysectomized animals had small tubules in which spermatogenesis did not proceed beyond the maturation division of spermatocytes (compare Figs. 15 and 17). Thyroxine effected no repair whatever, and in fact produced a decrease in the number of cells present in the seminiferous epithelium and a vacuolation in the Sertoli cytoplasm (Fig. 16, V).

Experiment C: Comparison of the effects of thyroxine in immature thyroidectomized and hypophysectomized male rats

The purpose of this experiment was to observe whether similar results would be obtained in young animals (45 gm.), since the disturbances in body growth or organ growth might be more striking.

For reasons that are not clear (too early weaning?) the intact animals showed only a slow growth. Even so, the trend of the findings in the various groups (Fig. 18) was similar to that observed in Experiment B. Thyroxine restored the growth of the thyroidectomized rats, but did not influence that of the hypophysectomized animals.

TABLE 5. INFLUENCE OF THYROXINE ON THE OXYGEN CONSUMPTION OF YOUNG THYROIDECTOMIZED AND HYPOPHYSECTOMIZED MALE RATS
(Experiment C)

Days after operation	Thyroidectomized control	Thyroidectomized +4 μg. thyroxine	Hypophysectomized control	Hypophysectomized +4 μg. thyroxine	Intact controls
8	76.7	100.5	60.7	77.0	82.4
9	74.1	99.3	65.4	81.0	88.4
14	70.5	107.6	68.8	82.8	85.8
Mean	73.7	102.4	64.9	80.2	85.5

The *oxygen consumption* (Table 5) was depressed in untreated thyroidectomized and even more in hypophysectomized animals. Thyroxine increased oxygen consumption to a somewhat higher level in thyroidectomized than in hypophysectomized rats.

The *organ weights* (Table 6) were generally restored to normal by thyroxine in thyroidectomized animals. The increase in weight produced by this hormone in hypophysectomized animals was less pronounced, although sufficient to bring the relative weights within normal range.

In the case of the testis, both tubules and interstitial cells were repaired by thyroxine in thyroidectomized animals. This hormone had no such effect in hypophysectomized animals.

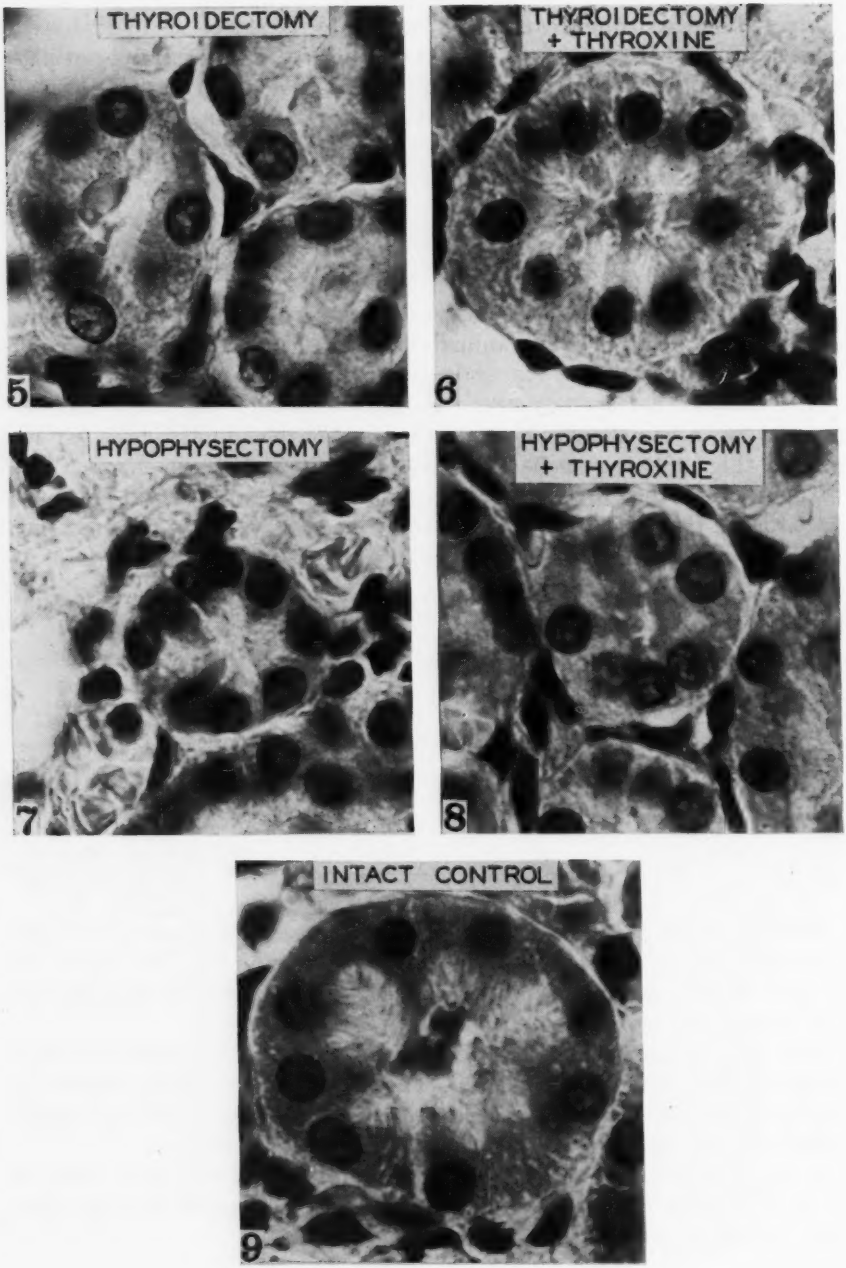

PLATE I (Figs. 5–9)

Experiment D: Effect of a 5-week thyroxine treatment on young adult hypophysectomized male rats

Experiment D was continued for 36 days, that is, longer than any of the other experiments, but on the whole, the results were the same as in Experiment A. The condition of the epidermis will be described in detail, since the prolongation of the experiment accentuated the findings in this tissue.

The *epidermis* was increased in thickness in the control hypophysectomized rats, in confirmation of previous observations (Leblond and Nelson, 1937). Here, the control hypophysectomized rats had received 0.2 mg. of cortisone daily, a dose which did not seem to change the appearance of the epidermis from that generally observed in untreated hypophysectomized rats. Thyroxine treatment, on the other hand, produced a pronounced decrease in the thickness of the epidermis (compare Figs. 19 and 20). This decrease was as clearcut as that observed in thyroidectomized animals treated with thyroxine for the same length of time (Eartly *et al.* 1951).

DISCUSSION

An effect of thyroxine has been defined above as the restoration by this hormone of a physiological process altered by thyroidectomy. Thyroxine effects are best detected by administration of a physiological dose to thyroidectomized animals. Such a dose in young adult rats weighing about 150 gm. lies between 3 and 6 μg. of thyroxine daily, for the oxygen consumption and heart rate of thyroidectomized animals given these amounts were either slightly smaller or greater respectively than those observed in intact controls (Figs. 3 and 4).

When the processes restored by such doses of thyroxine in thyroidectomized rats were investigated in similarly treated hypophysectomized animals, they were found to be either 1) completely restored, 2) absent, or 3) partially restored.

Explanation of Plate I

Influence of thyroxine on the proximal convoluted tubules of the kidney in thyroidectomized and hypophysectomized male rats.

Fig. 5. Thyroidectomy. Note the relatively small size of tubules when compared with those of intact control animals (Fig. 9).

Fig. 6. Thyroidectomy with thyroxine treatment (6 μg. daily). The tubule size is restored to normal (compare with Fig. 9).

Fig. 7. Hypophysectomy. The tubule size is smaller than in thyroidectomized animals (compare with Fig. 5).

Fig. 8. Hypophysectomy with thyroxine treatment (6 μg. daily). The tubule size is only slightly increased by thyroxine.

Fig. 9. Intact control.

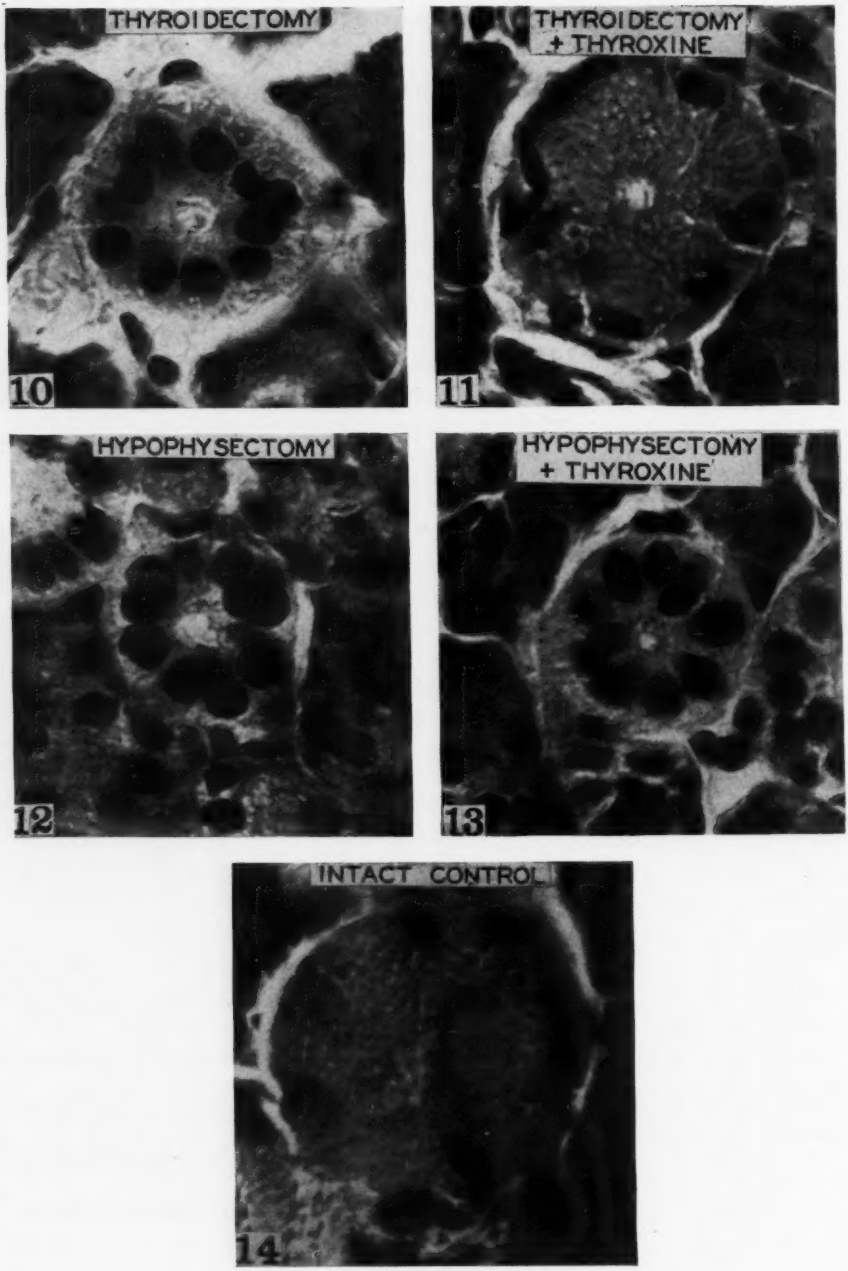

PLATE II (Figs. 10–14)

1. *Physiological processes completely restored by thyroxine in hypophysectomized animals*

Doses of 3 or 6 μg. of thyroxine raised the *heart rate* of hypophysectomized rats (as well as of thyroidectomized rats) from the low level observed after operation to the normal range (Fig. 4).

The same doses of thyroxine also raised the *oxygen consumption* in both hypophysectomized and thyroidectomized rats (Fig. 3; Tables 2 and 5), in confirmation of the observations of Foster and Smith (1926), Reiss and Fischer-Popper (1936) and Basténié and Kowalewski (1951). However, the oxygen consumption reached a higher level in thyroidectomized than in hypophysectomized rats with 3 or 6 μg. of thyroxine in Experiment B (Fig. 3) and with 4 μg. of thyroxine in Experiment C (Table 5). This may be related to the fact that, in the absence of thyroxine treatment, the oxygen consumption was also significantly greater in thyroidectomized than in hypophysectomized animals (Fig. 3). On the other hand, with 12 μg. of thyroxine, the response reached the same level in both types of animals, and therefore the initial difference had been overcome.

Prolonged thyroxine treatment of hypophysectomized animals produced a *thinning of the epidermis* (compare Figs. 19 and 20) identical to that formerly observed in thyroidectomized-castrated rats treated with thyroxine for 5–6 weeks (Eartly *et al.* 1951). These authors showed that thinning was the ultimate result of a decrease in mitotic activity which in turn caused fewer epidermal cells to be formed.

Since these three effects of thyroxine, namely, accelerated heart rate, heightened oxygen consumption, and thinning of the epidermis could be obtained in the absence of the hypophysis, it was concluded that they were produced by a mechanism independent of the secretions of this gland.

In fact, this mechanism was not hormonal at all, since the three effects were obtained after extirpation of adrenals and testes (Eartly, 1951), or parathyroids (which had been removed with the thyroids in the thyroid-

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EXPLANATION OF PLATE II

Influence of thyroxine on the serous tubules of the submaxillary gland of thyroidectomized and hypophysectomized male rats.

FIG. 10. Thyroidectomy. Note small size of tubules and complete absence of granules.

FIG. 11. Thyroidectomy with thyroxine treatment (6 μg. daily). Both tubule size and granule content are nearly normal (cf. Fig. 14).

FIG. 12. Hypophysectomy. Note small size of tubules and complete absence of granules.

FIG. 13. Hypophysectomy with thyroxine treatment (6 μg. daily). Thyroxine did not increase the size of tubules nor did it restore the granules.

FIG. 14. Intact control.

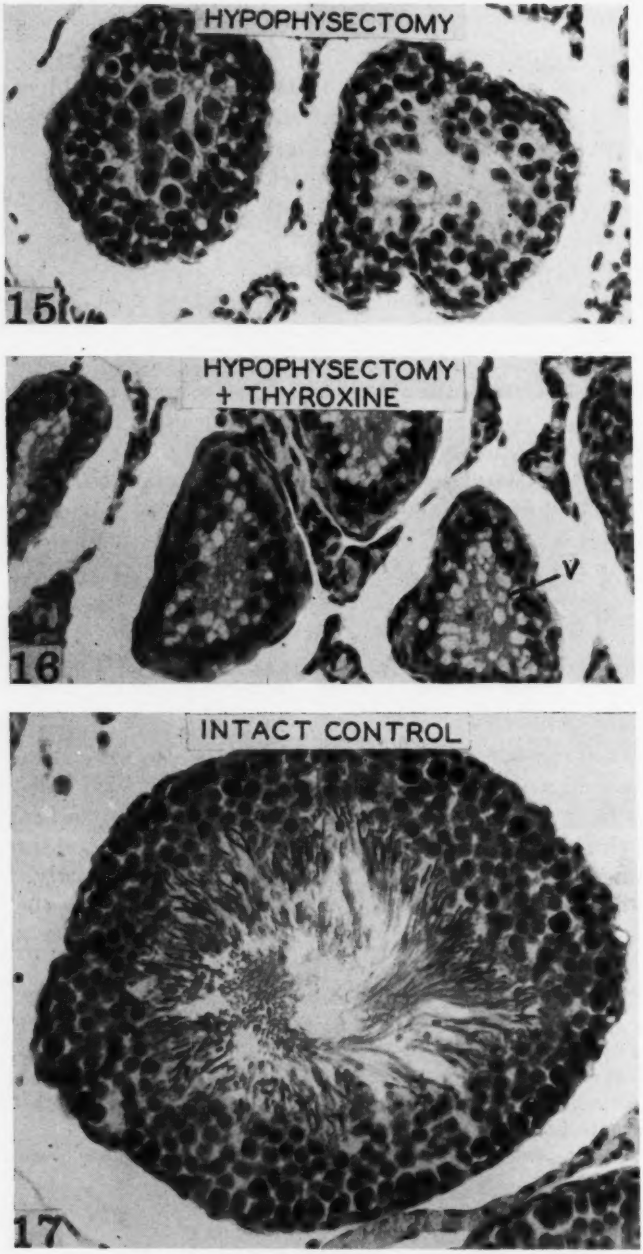

PLATE III (Figs. 15–17)

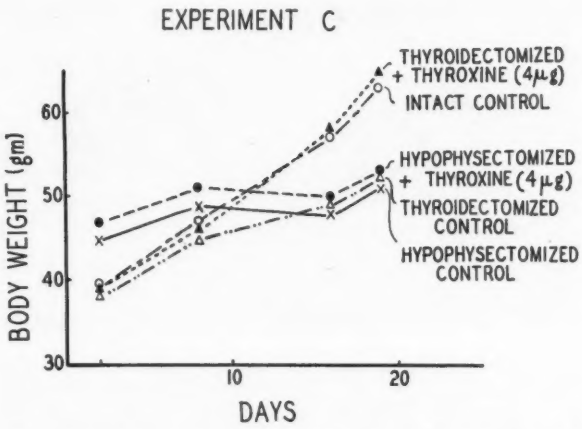

FIG. 18. Influence of thyroxine on the body weight of immature thyroidectomized and hypophysectomized male rats. The hypophysectomies were carried out on Day 0 and the thyroidectomies on Day 2. Treatment with thyroxine was started on Day 1.

Thyroidectomized animals gained weight at a reduced rate. Hypophysectomized animals showed a greatly reduced growth rate immediately after operation. Whereas thyroxine restored growth to normal in thyroidectomized rats, it was ineffective in hypophysectomized rats.

ectomized animals of Experiments B, C and D). The possibility that the islets of Langerhans could play a role appeared unlikely since insulin, unlike thyroxine, does not possess the ability to raise the basal metabolic rate (Selye, 1947). Thus, these effects of thyroxine are not mediated by other hormones; and, therefore, from the endocrine point of view, they may be referred to as *direct*.

Acceleration of the heart rate, like increased oxygen consumption, is a manifestation of thyroxine's overall activation of metabolism (Leblond and Hoff, 1944). Furthermore, an increase in metabolic rate is associated with glycogen utilization and a lowering of the glycogen stores (McDonald, Boyle and de Groat, 1938; Sternheimer, 1939; Moses, 1944), a situation resulting in a lowering of mitotic activity (Bullough and Eisa, 1950) and

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EXPLANATION OF PLATE III

Influence of thyroxine on the seminiferous tubules of hypophysectomized rats.

FIG. 15. Hypophysectomy. Note small size of tubules and arrest of spermatogenesis.

FIG. 16. Hypophysectomy with thyroxine treatment (6 μg. daily). Note small size of tubules, arrest of spermatogenesis, and the presence of vacuoles in the Sertoli cytoplasm (V).

FIG. 17. Intact control.

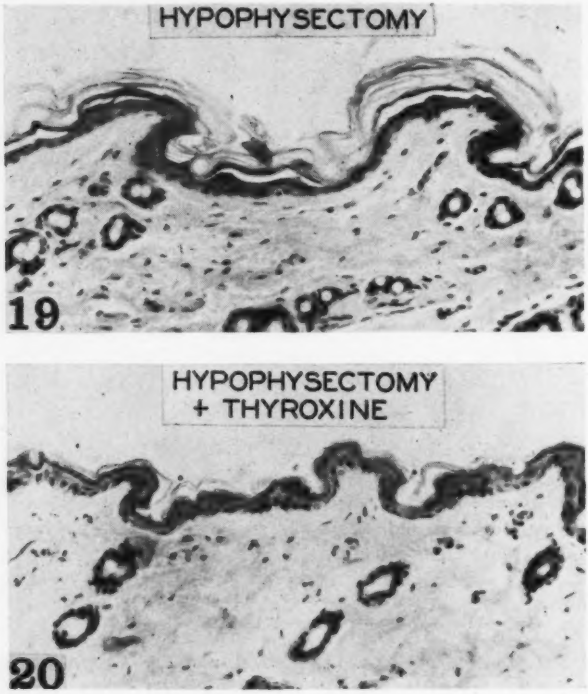

PLATE IV (Figs. 19 and 20)

Influence of thyroxine on the epidermis of hypophysectomized male rats.

The thickness of the cornified layers is greater in the hypophysectomized controls (Fig. 19) than in those given thyroxine (Fig. 20).

this in turn is believed to account for the thinning of the epidermis observed after thyroxine (Eartly *et al.* 1951).

Thus, the three effects of thyroxine which were present in hypophysectomized animals appeared to be results of metabolic activation—perhaps the most fundamental action of this hormone. The speed-up of many physiological and biochemical responses noted after thyroid treatment (Salter, 1950) may also be due to a heightened metabolic rate and thus belong in this category.

2. *Physiological processes absent in hypophysectomized rats treated with thyroxine*

The growth promoting effect of thyroxine may be shown by the fact that the *body weight* plateaued after thyroidectomy (Evans *et al.*, 1939), while a normal rate of increase in body weight was maintained by thyrox-

ine administration (Fig. 2). However, thyroxine had no significant influence on the body weight of hypophysectomized rats (Fig. 2), in confirmation of previous work by Smith (1927), Smith *et al.* (1927), Evans *et al.* (1939), and Weisschedel (1950, a, b). Nevertheless, a small effect of thyroxine on the body weight of hypophysectomized animals was observed by Ray *et al.* (1950) and particularly by J. Salter and C. H. Best (private communication). Salter suggested that the discrepancy between his and the present results might be due to differences in the diet. At any rate, in our work, thyroxine increased body weight only when the hypophysis was present. Thus, the body weight increasing effect of thyroxine required the presence of the hypophysis.

The role of the thyroid gland in *testis growth* was examined in the young thyroidectomized rats of Experiment C (Table 6). The testes were atrophic in untreated hypophysectomized rats. They were only slightly larger in untreated thyroidectomized rats and, therefore, little growth of the testis took place in the absence of thyroid hormone. (In older animals, however, unpublished experiments revealed that the role of thyroxine in the maintenance of testis size was less important.) On the other hand, administration of thyroxine to hypophysectomized animals did not repair, but even damaged the testis since the weight of this organ decreased further and Sertoli elements showed vacuolation (Fig. 16), while the same hormone in thyroidectomized rats restored the testis to the normal range of weight and histological appearance. Apparently, the presence of the hypophysis in these animals had enabled thyroxine to induce a normal testicular development (and thus completely overcame any damaging influence that this hormone might have).

The influence of graded doses of thyroxine on the *adrenal gland* was to produce a graded increase in size of these glands in thyroidectomized animals as shown by their absolute weights in Experiment B (Table 4). This effect of thyroxine was completely absent in the corresponding groups of hypophysectomized animals (Table 4; see also Table 3); and, therefore, this effect required the presence of the hypophysis.

Thus, thyroxine promotes body growth, as well as growth of testis and adrenal, but only in the presence of the hypophysis.

In considering how the hypophysis mediates these actions of thyroxine, it may first be noted that the effect of this hormone on *body growth* has been attributed to the release of hypophyseal growth hormone by Scow *et al.*, (1949), Koneff *et al.*, (1949), and Becks *et al.* (1950). The absence of pituitary acidophils—presumably the sites of growth hormone synthesis and secretion (Severinghaus, 1939)—would explain the lack of growth observed in thyroidectomized rats, while maintenance of the acidophils by thyroxine replacement would result in a secretion of growth hormone by these cells

TABLE 6. INFLUENCE OF THYROXINE ON THE ORGAN WEIGHTS OF YOUNG THYROIDECTOMIZED AND HYPOPHYSECTOMIZED MALE RATS
(Experiment C)

	Operated control		4 μg. thyroxine		Intact control
	T-ect.	Hy-ect.	T-ect.	Hy-ect.	
Final number of animals	18	6	15	5	10
Initial body weight (gm.)	38	45	39	47	39
Final body weight (gm.)	52	51	65	53	63 / 63
Organ weights					
Absolute (mg.)					
Relative (mg./100 gm. body weight)					
Kidneys	441	429	766	587	716 / 716
	859	*819*	*1187*	*983*	*1102 / 1102*
Heart	216	208	322	260	254 / 254
	413	*400*	*501*	*487*	*403 / 403*
Thymus	139	107	139	131	126 / 126
	269	*203*	*208*	*245*	*196 / 196*
Spleen	325	330	330	292	336 / 336
	625	*647*	*507*	*551*	*533 / 533*
Liver	2183	1738	2547	1992	2473 / 2473
	4155	*3308*	*3892*	*3660*	*3905 / 3905*
Salivary glands	280	214	233	183	287 / 287
	538	*419*	*358*	*345*	*455 / 455*
Adrenals	14	8	18	8	14 / 14
	26	*15*	*20*	*16*	*22 / 22*
Testes	331	109	680	84	756 / 756
	636	*272*	*1040*	*165*	*1200 / 1200*
Seminal vesicles	10	10	10	14	12 / 12
	18	*19*	*15*	*24*	*19 / 19*
Thyroid		5.9 / *11.5*		7.7 / *14.5*	8.6 / *13.6*
Pituitary	3.1 / *5.9*		3.0 / *4.6*		2.4 / *3.8*

Any underlined figure is significantly different from that of the operated control group on the left of the same line.

and, as a consequence, an increase in body weight. In the case of the *testis*, the administration of thyroxine to thyroidectomized animals restored not only weight but also the histological appearance of tubules and interstitial cells. A similar repair of the testis can be produced in hypophysectomized animals only by administration of gonadotrophic hormones (Greep and Fevold, 1937; Nelson, 1937). The release of such hormones by the hypophysis of thyroidectomized rats under the influence of thyroxine would account for testicular growth. Finally, the increase in size of the *adrenal gland* may be attributed to the release of ACTH (Noble, 1950) occurring under the influence of thyroxine.

The complete absence of *body growth* in untreated thyroidectomized rats by the end of the second post-operative week (Fig. 2) suggested a complete interruption of growth hormone secretion, whereas the limited *growth of the testis* suggested that little gonadotrophic material was available. Thus, in the absence of thyroid hormone, the hypophysis would secrete no growth hormone and little gonadotrophic hormone. The case was rather different with the *adrenal*, as this organ was found to be as large in thyroidectomized animals as in intact controls in both Experiments B (Table 4) and C (Table 6). A decrease in adrenal size after thyroidectomy was, however, described by Freedman and Gordon (1950), Feldman (1951) and others. It was possible that, in the thyroidectomized rats of Experiment B such a decrease was masked by an opposite effect of testis extirpation (Lawless, 1936), as the testes of these animals had also been removed (Table 1). The effect of thyroidectomy on the adrenal should be investigated further. In any case, the fact that thyroxine increased the adrenal weight in thyroidectomized but not in hypophysectomized animals (Table 4) suggested that thyroxine augments the release of corticotrophic hormone. Finally, with regard to other pituitary hormones, it is also known that the increase in thyrotrophic secretion which occurs after thyroidectomy is inhibited by thyroxine (Halmi, 1952). It may be concluded that, under the influence of thyroxine, the hypophysis reduces its secretion of thyrotrophic hormone, but increases that of growth, gonadotrophic, and corticotrophic hormones. Thus, thyroxine plays a role in the control of a number of pituitary secretions.

3. *Physiological processes partially restored by thyroxine in hypophysectomized animals*

The splanchnic enlargement due to the thyroid hormone (Herring, 1917) was seen here as a pronounced increase in the *weight of non-endocrine organs* in thyroidectomized rats and a smaller, though still significant, enlargement in hypophysectomized rats (Tables 4 and 6).

These results suggest that the organ growth due to thyroxine has at

least two components: 1) one detected in hypophysectomized animals and contributing to the growth of the organs a share such as to restore their relative weight; 2) one seen in thyroidectomized animals and insuring a complete restoration of growth since the absolute weights of most organs were the same as in intact controls. It is presumed that the growth component detected in hypophysectomized animals is not mediated by other hormonal secretions and, in this sense, may be described as *direct*, while the completion of organ growth observed in thyroidectomized animals is a *hypophysis-mediated* action of thyroxine.

The *direct* component of thyroxine's effect on organ weight may be a result of thyroxine's overall metabolic activation. When graded doses of thyroxine stimulate the metabolism to increasing levels, a graded rise in heart rate associated with a graded hypertophy of the heart occurs (despite a marked loss in body weight in the animals given the largest doses; Leblond and Hoff, 1944; Leblond, 1944). These authors also observed a graded enlargement of other organs, such as kidney or liver. It is believed that in all these organs the extra load due to increased metabolism caused an increase in size, i.e., a functional hypertrophy.

The *hypophysis-mediated* component of the effect of thyroxine on organ growth was, like body growth, observed in thyroidectomized animals only. Since the work of Evans and his associates quoted above indicated that growth hormone is released by the hypophysis under thyroxine influence and, furthermore, since this hormone is known to increase organ weights in normal and hypophysectomized rats (Selye, 1947), release of growth hormone due to the influence of thyroxine would provide the hypophyseal mediator postulated to explain the second component.

This dual interpretation of thyroxine effect on organ growth accounts for the observations of Simpson *et al.* (1949) that growth hormone, although greatly increasing the total body weight of hypophysectomized rats, did not restore the proportionate weight of heart, kidneys, intestine, brain, and eyeballs. It also supports the data of Scow *et al.* (1949) which showed that in very young thyroidectomized rats growth hormone and thyroxine given simultaneously had an additive effect on organ weights.

Histologically, the combination of direct and hypophysis-mediated effects was also recognizable. Thus, the proximal convoluted tubules of the kidney were restored by thyroxine to a small extent in hypophysectomized and completely in thyroidectomized rats (Figs. 5–9). Unpublished work by R. Carriere in which the diameter of the proximal tubules was measured generally confirmed these conclusions.

The serous granules in the serous tubules of the *submaxillary gland* were fairly well restored by thyroxine in thyroidectomized but not in hypo-

physectomized animals. Even the combination of thyroxine and testosterone, which was fully effective after thyroidectomy (Grad and Leblond, 1949), had no effect whatever in hypophysectomized animals (Eartly, 1953). The serous granules would, therefore, appear to be under the direct control of a hypophyseal factor.

Finally, in the case of the *thymus*, a growth-promoting effect of thyroxine was recognizable in hypophysectomized animals with the 3 and 6 μg. doses but not with the toxic 12 μg. dose.

However, it should be emphasized that the main conclusions of this work were reached by considering results obtained with doses of thyroxine which lie within the physiological range. Since thyroxine may be considered to be a thyroid hormone (Leblond, 1951), these conclusions apply to the physiological role of the thyroid gland. Thus, this gland would appear to

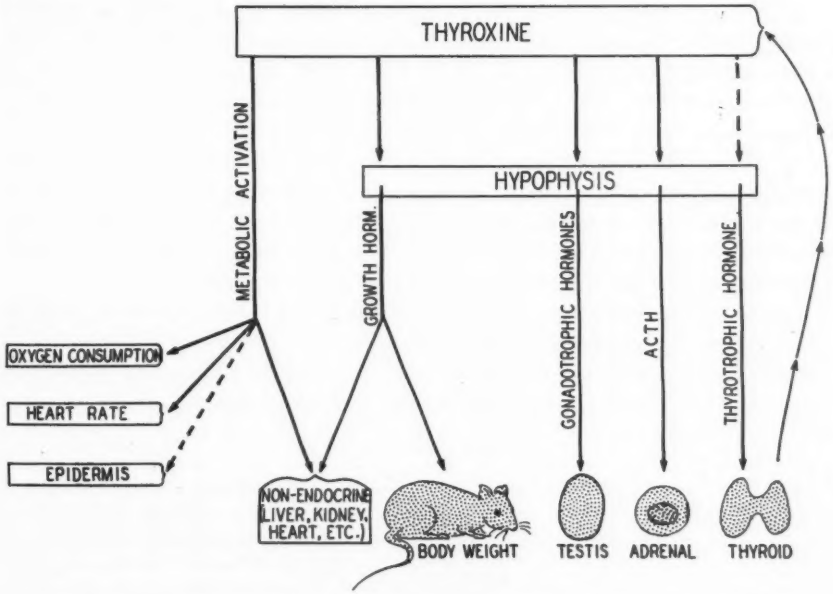

FIG. 21. Diagram summarizing the interpretation of the stimulating (solid lines) and inhibiting (broken lines) effects of physiological doses of thyroxine.

On the left, the metabolic activation due to thyroxine is shown to influence oxygen consumption, heart rate, epidermis, and in part organ weight.

The middle portion of the diagram indicates that thyroxine stimulates the hypophysis to secrete growth hormone (which affects organ weight as well as general body growth), gonadotrophic hormones, and ACTH, while inhibiting to some extent the release of thyrotrophic hormone.

have a *direct* effect on metabolism, a *hypophysis-mediated* effect on body growth, adrenals, and male sex glands, and a *combination* of direct and hypophysis-mediated effects on the size of non-endocrine organs (Fig. 21).

SUMMARY

Physiological doses of thyroxine were administered to male rats of various ages after either thyroidectomy or hypophysectomy and the resulting biological responses were compared.

Three categories of thyroxine effects have been found, namely:

1) Those which occur in the absence of the hypophysis, that is, increase in oxygen consumption, acceleration of heart rate, and thinning of the epidermis. Such effects are believed to be the result of the overall metabolic activation due to thyroxine. Available evidence indicates that thyroxine produces this activation by a direct effect on non-endocrine tissues.

2) Those which do not occur in the absence of the hypophysis, namely, body growth, sexual development, and adrenal growth. These three effects of thyroxine are attributed to the fact that thyroxine exerts some control on the release from the hypophysis of growth, gonadotrophic, and corticotrophic hormones respectively.

3) Those which show partial restoration in the absence of the hypophysis, in particular, splanchnic enlargement. Such an effect is attributed in part to a direct metabolic action of thyroxine itself, in part to mediation via a pituitary factor such as growth hormone. Both factors would combine to produce the organ growth induced by thyroxine.

In conclusion, maintenance of the normal condition of several physiological functions (basal metabolism, heart rate, etc.) and morphological structures (kidney, lymphatic system, testis, etc.) has been shown to depend on direct and/or hypophysis-mediated effects of thyroxine.

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SOME BIOLOGICAL PROPERTIES OF AMINOSTEROIDS

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THIS report concerns some biological properties of a steroid, 17-amino-4-androsten-3-one monohydrochloride, designated by the code number, Su-1621.[2] A few preliminary observations are included on a related steroid, Su-1092, 20-amino-3β-hydroxy-5-pregnene monohydrochloride. Su-1621, unlike testosterone of which it is an amino derivative, was found to have essentially no effects on the reproductive tract. Further studies showed that it had, nevertheless, definite biological actions in female but not male rats. Among these effects were a remarkable increase in kidney weight and lesser increases in liver and adrenal weight together with a decrease in thymus weight, growth rate and appetite. Subsequently, when it is said that animals were responsive to the compound the responsiveness was judged by this quintet of effects of which the change in kidney weight was always the most striking and reliable.

Dr. C. Riley Houck of the University of Tennessee has studied the effects of Su-1621 in dogs with particular relation to kidney function. His work, which will be published later, shows that there are marked differences in the response of the rat and the dog to the compound.

METHODS

Su-1621, being a hydrochloride of an amine, was highly soluble in water and hence was used routinely in aqueous solution at a concentration of 10 mg. per ml. in single daily doses. It was generally given subcutaneously but was equally effective when given by stomach tube.[3] In most cases adult rats received 4 mg. of the compound daily for 10 days, as indicated in the tables by "4 mg./day×10." Autopsies were done 24 hours after the last injection, i.e., usually on the eleventh day. The rats used were from an inbred strain, originally of Wistar stock. In all cases the statistical value included is the standard error of the mean: $\sqrt{\Sigma d^2/n(n-1)}$.

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[1] From the Department of Zoology, Rutgers University.

[2] Dr. H. B. MacPhillamy and his associates of the Chemical Research Division of this laboratory prepared Su-1621 and Su-1092 for this work. The authors are also indebted to Dr. E. Oppenheimer for advice and suggestions throughout the study and to Miss Gloria J. Miller and Mr. Ben Antonchak for technical assistance.

[3] In one series of animals the effects of Su-1621 were not enhanced by suspending the crystals in sesame oil for subcutaneous injection as compared with other means of administration.

Non-effect of Su-1621 on the reproductive tract

In a variety of observations not described here in detail, involving both acute and chronic administration of Su-1621 at different dose levels, no evidence was obtained of an effect on the weight of ovaries, testes, uteri, seminal vesicles, or pituitary glands. With high doses estrous cycles became somewhat irregular. This was probably a manifestation of the general toxic action of the compound.

The effect of Su-1621 on organ weights in female rats (Table 1)

Series 1, 2, and 3 show an approximate doubling of kidney weight when 2 or 4 mg. of Su-1621 were given per day for ten days. The characteristic increase in liver and adrenal weight and decrease in thymic and body weight is also evident in these tables. Series 4–7 show dose-response relations and indicate that 1 mg. per day for five days

TABLE 1. EFFECT OF Su-1621 ON BODY AND ORGAN WEIGHTS IN RATS

Ser. No.	No. rats	Treatment	Body weight, gm.			Kidney wt., gm./100 gm.	Liver wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.
			Init.	Term.	% Ch.				
1	28	Mature ♀ Controls—untreated	231	236	+ 2	0.73±0.103	3.7±0.060	25.0±0.54	130± 6.8
2	16	Mature ♀; Su-1621, 2 mg./day ×10	205	206	0	1.30±0.048	4.7±0.083	25.8±0.77	111±12.2
3	32	Mature ♀; Su-1621, 4 mg./day ×10	233	209	−10	1.80±0.057	4.7±0.058	30.6±0.86	57± 3.5
4	10	Immature ♀ Controls—untreated	50	74	+48	1.07±0.028	4.5±0.083	21.3±0.70	424±29.7
5	10	Immature ♀; Su-1621, 1 mg./day ×5	51	73	+43	1.05±0.032	4.4±0.062	22.2±0.82	399±22.7
6	10	Immature ♀; Su-1621, 2 mg./day ×5	48	53	+10	2.09±0.194	4.9±0.128	32.9±2.68	237±38.4
7	10	Immature ♀; Su-1621, 4 mg./day ×5	50	49	− 2	3.49±0.310	6.7±0.364	45.2±4.03	112±14.5
8	14	Mature ♂ Controls—untreated	219	269	+23	0.80±0.014	4.1±0.062	14.4±0.35	176± 8.6
9	17	Mature ♂; Su-1621, 4 mg./day ×10	210	241	+15	0.80±0.014	4.3±0.093	14.8±0.56	174±10.1
10	9	Immature ♂ Controls—untreated	45	97	+116	1.19±0.046	5.2±0.240	19.8±1.12	385±16.0
11	15	Immature ♂; Su-1621, 2 mg./day ×10	47	87	+85	1.27±0.073	5.5±0.121	23.4±1.47	329±18.8

was without effect on immature female rats, while higher doses produced effects like those seen in adult animals. Injections of the higher doses could not be continued uniformly for 10 days as in adults because of the toxicity of the compound.

Effects of Su-1621 on organ weights in male rats

In striking contrast to the results in females, neither mature nor immature male rats showed more than slight if any evidence of effects from the administration of Su-1621 (Table 1, Series 8–11).

Effect of castration and gonadal hormones on response of mature animals to Su-1621

A variety of experiments were undertaken to determine whether the greater responsiveness of female than male animals to Su-1621 could be explained on an endocrine basis. The responsiveness to the compound after castration is shown in Table 2. Su-1621 was given during the last ten days of the castration periods. In Series 12 and 13 it is apparent that ovariectomy for as long as 40 days did not abolish nor apparently diminish responsiveness to Su-1621. Castration in the male for 14 days did not abolish the relative refractoriness of that sex to the compound (Series 14 and 15).

TABLE 2. EFFECT OF CASTRATION OF MATURE ANIMALS ON RESPONSIVENESS TO Su-1621

Ser. No.	No. rats	Treatment	Body weight, gm. Init.	Term.	% Ch.	Kidney wt., gm./100 gm.	Liver wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.
12	6	♀—Castrate 40 days—Control	266	282	± 6	0.66±0.018	3.5±0.103	21.0±1.74	178± 8.5
13	10	♀—Castrate 40 days; Su-1621, 4 mg./day×10	275	254	± 8	1.22±0.050	4.0±0.066	25.2±0.31	109± 6.9
14	6	♂—Castrate 14 days—Control	186	223	±20	0.78±0.063	4.2±0.130	18.8±2.07	233±15.1
15	6	♂—Castrate 14 days; Su-1621, 4 mg./day×10	187	212	±13	0.90±0.071	4.5±0.148	18.4±1.50	196±16.3

TABLE 3. EFFECT OF ESTRADIOL BENZOATE ON RESPONSIVENESS OF MATURE MALES TO Su-1621

Ser. No.	No. rats	Treatment	Body weight, gm. Init.	Term.	% Ch.	Kidney wt., gm./100 gm.	Liver wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.
16	6	Su-1621, 4 mg./day×10; Est. benz., 1γ/day×10	194	212	+ 9	0.87±0.025	4.5±0.589	19.1±0.44	156± 9.0
17	6	Su-1621, 4 mg./day×10; Est. benz., 10γ/day×10	206	197	− 4	1.46±0.142	4.7±0.156	23.4±0.11	91±20.6
18	6	Est. benz., 10γ/day×10	201	228	+13	0.78±0.017	4.3±0.064	18.8±0.109	176±27.1
19	6	Est. benz., 10γ/day×10; followed by Su-1621, 4 mg./day×10	188	164	−13	2.43±0.21	5.0±0.132	33.4±0.235	73±9.7
20	3	Est. benz., 10γ/day×10; after 5 days Su-1621, 4 mg./day×10	278	237	−15	1.85	4.7	25.9	75
21	3	Est. benz., 10γ/day×10; after 10 days Su-1621, 4 mg./day×10	251	223	−11	1.78	4.0	25.2	111

Effect of gonadal hormones

The administration of 1 μg./day of estradiol benzoate had no effect on the response of mature males to Su-1621 (Table 3, Series 16). In series 17–21, however, it is apparent that 10 μg. of estradiol benzoate administered with Su-1621, or for various periods preceding it, resulted in responsiveness of males roughly equal to that seen in normal females.

In view of the above findings it was of interest to learn whether the administration of testosterone propionate would block responsiveness to Su-1621 in adult female animals. In Table 4, Series 22–25, it is seen that when testosterone propionate, at either of two dose levels, was given concomitantly with Su-1621 it did not modify in any apparent way the response to the latter.

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE ON RESPONSIVENESS OF MATURE FEMALES TO Su-1621

Ser. No	No. rats	Treatment	Body weight, gm. Init.	Term.	% Ch.	Kidney wt., gm./100 gm.	Liver wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.	Food consumption, gm./day/rat
22	10	Controls, untreated	188	208	±10	0.87±0.24	4.5±1.12	29.0±2.08	199± 8.1	21.2
23	9	Su-1621, 4 mg./day×10	188	173	± 8	2.47±0.14	5.4±0.40	38.0±2.29	79±12.2	12.2
24	10	Su-1621, 4 mg./day×10; T.P., 0.5 mg./day×10	190	178	± 7	2.26±0.48	5.1±0.09	28.6±1.70	46± 5.2	11.0
25	10	Su-1621, 4 mg./day×10; T.P., 1.0 mg./day×10	180	173	± 4	2.18±0.11	5.1±0.33	31.8±2.03	72± 7.6	12.1

TABLE 5. EFFECT OF PRE-PUBERAL CASTRATION WITH AND WITHOUT SEX HORMONE TREATMENT ON RESPONSIVENESS TO Su-1621

(All animals castrated at 22–24 days of age and autopsied 39 days later; sex hormone treatment given during entire period; Su-1621 given during last 10 days only)

Ser. No.	No. rats	Treatment	Body weight gm.			Kidney wt., gm./100 gm.	Liver wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.
			Init.	Term.	% Ch.				
26	9	Female, untreated	53	194	+266	0.79±0.014	4.1±0.172	23.8±1.07	321±19.3
27	5	Female, Su-1621, 4 mg./day×10	50	142	+148	1.94	5.0	31.8	141
28	3	Male, untreated	43	217	+404	0.86	4.4	17.8	316
29	5	Male, Su-1621, 4 mg./day×10	45	187	+315	1.01	5.0	21.4	259
30	9	Female, T.P., 0.1 mg./day ×39	54	213	+294	0.87±0.017	4.3±0.137	15.7±0.696	166±12.6
31	15	Female, T.P., 0.1 mg./day ×39; Su-1621, 4 mg./ day×10	51	197	+286	0.88±0.014	4.6±0.086	16.6±0.60	136±10.6
32	4	Male, Est. benz., 1γ/day ×39; Su-1621, 4 mg./ day×10	45	150	+233	2.06	6.1	31.7	117

Effect of pre-puberal castration with and without gonadal hormone treatment on response to Su-1621 (Table 5)

In view of the negative results with testosterone propionate mentioned above, a series of experiments were set up to determine the effect of pre-puberal castration at 22–24 days of age and of early and long-continued administration of gonadal hormones on the sex difference in sensitivity to Su-1621. The results in treated animals, along with appropriate controls, are shown in Table 5.

The organs studied were not clearly affected by castration alone (Series 26 and 28). It is apparent from Series 27 that if the female castrate was given Su-1621 during the last ten days of a 39-day castration period the response to Su-1621 is similar or identical to that which occurs in intact females. Although the number of cases on which to base judgment is undesirably small, it is probable that the pre-puberally castrate male, like the normal or adult castrate, was not responsive to Su-1621 (cf. Table 1, Series 10 and Table 5, Series 28 and 29).

On the other hand, it was entirely clear that testosterone given to females from the time of pre-puberal castration blocked the responsiveness to Su-1621 (cf. Series 26, 27 and 30). This result is in contrast to that obtained when testosterone was given to adult females for shorter periods (Table 4, Series 22–25).

As was true in adult intact animals, full responsiveness to Su-1621 occurred after long-term treatment with estradiol benzoate in the male castrated prepuberally (Series 32).

Effect of adrenalectomy on the response to Su-1621

Preliminary experiments showed that Su-1621 reduced the life-span of adrenalectomized rats which were otherwise untreated. This made it difficult to obtain a uniform series treated only with Su-1621 for the same length of time. In all cases, however, adrenalectomized female animals treated with Su-1621 even for short periods showed increases in kidney weight.

When 25 μg. per day of DCA was given with Su-1621 better survival resulted and the nephromegalic action of the latter was apparent (Table 6). It is concluded that most of the effects of Su-1621 are not mediated through the adrenals.

The fact that relatively slight thymic atrophy occurred in the adrenalectomized rats

TABLE 6. EFFECT OF ADRENALECTOMY ON RESPONSIVENESS TO Su-1621
(All animals received 25γ/day of DCA)

Ser. No.	No. Rats	Treatment	Body weight, gm.			Kidney wt., gm./ 100 gm.	Liver wt., gm./ 100 gm.	Thymus wt. mg./ 100 gm.
			Init.	Term.	% Ch.			
33	5	DCA only	56	83	+48	1.17	4.5	517
34	5	Su-1621, 4 mg./day ×10 plus DCA	54	72	+33	2.02	4.9	438

receiving Su-1621 (cf. Tables 1 and 6), indicates, on the other hand, that the loss of thymic weight usually seen in intact or castrate animals was due to adrenal hyperactivity.

Toxic effects of Su-1621

As is evident from all of the tables, one of the consistent effects of high doses of Su-1621 in responsive animals was either to decrease the growth rate or to cause an actual loss of body weight. This effect was associated with a marked decrease in food intake. The latter is shown in Table 4, in which the food intake was determined simply by measuring the amount of food pellets which "disappeared" from feed baskets during the treatment period without regard for waste. If one could judge by the results of the more precise paired-feeding experiments shown in Tables 7 and 8, the weight loss during Su-1621-treatment could be accounted for entirely by decreased appetite. The data, however, are not sufficient for final conclusions on that point.

Su-1621 frequently caused local necrosis at subcutaneous injection sites a few days after the injections were made. The acidity of the solution injected (pH, 4.6) was not sufficient to account for this effect. This fact precluded long-term subcutaneous administration, a problem which in turn could be circumvented by giving the compound by stomach tube.

Intravenous (tail vein) administration of Su-1621 in doses of 3–4 mg./100 gm. caused convulsions and death in approximately half of the adult animals of either sex. No precise determinations of lethal doses, however, were made. This was the only instance observed in which male and female animals responded alike to Su-1621. No evidences of steroid anesthesia were seen.

Our colleague, Dr. Albert Plummer, determined that Su-1621 caused hemolysis of dog erythrocytes *in vitro* at a concentration of 1–10 mg./ml. but not at 0.5 mg./ml. Such lytic action may be correlated with the irritating action of the compound at injection sites.

When young, female rats were given as much as 5 mg. of Su-1621 subcutaneously at a single dose, convulsions of an unusual sort occurred in about half of the cases

TABLE 7. EFFECT OF Su-1621 ON WATER AND PROTEIN CONTENT OF LIVER AND KIDNEY OF PAIR-FED FEMALE RATS

No. rats	Treatment	Kidney				Liver				Body change wt. %
		Wt. gm./100 gm.	% Water	% Protein of dry wt.	Total protein, mg.	Wt. gm./100 gm.	% Water	% Protein of dry wt.	Total protein, mg.	
6	Controls, pair-fed	0.67± 0.023	74.4± 0.6	66.6± 0.088	238.7± 5.24	2.33± 0.069	70.0± 0.038	68.5± 1.06	1036± 32.2	−9
6	4 mg. Su-1621/day ×12	1.28± 0.058	82.8± 1.86	59.8± 3.01	266.5± 27.62	3.49± 0.289	72.7± 1.120	70.1± 0.84	1507± 89.9	−5

TABLE 8. EFFECT OF SU-1621 ON HEPATIC REGENERATION AFTER PARTIAL HEPATECTOMY IN FEMALE RATS

No. rats	Body weight, gm.			Amt. liver excised, gm./100 gm.	Wt. regenerated liver, gm./100 gm.	Liver dry wt., gm./100 gm.	Kidney wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.
	Init.	Term.	% Ch.						
Controls—No treatment after partial hepatectomy									
12	217	184	−15	2.56±0.056	2.77±0.025	0.81*	0.77±0.025	31.7±1.53	108±5.8
Su-1621, 4 mg./day ×4, after partial hepatectomy									
9	221	188	−15	2.59±0.060	3.80±0.69	0.96*	1.59±0.070	44.3±2.40	88±7.5

* 6 cases only.

within 15 to 45 minutes. These consisted of sudden seizures, generally of about 30 seconds' duration, characterized by generalized clonic twitching and often ending with the rat squatting on its hind legs and pawing the air with the forelegs. The seizures were intermittent, of variable number and frequency, and only rarely resulted in death. No systematic effort was made to characterize this phenomenon pharmacologically.

Effects of Su-1621 on the kidney and liver

Histological studies were made on kidneys removed from animals after a 10-day period of treatment with 4 mg./day of Su-1621. These kidneys showed consistently a hydronephrosis in which swollen tubules at times contained leucocytes while lacking eosinophilia. On occasion the tubular lumina were obliterated. Interstitial fibrosis was generally present. Infrequent tubular mitoses were observed. The damage was apparently permanent: at two months following a 10-day period of treatment an extensive, densely cellular interstitial fibrosis was present. Occasionally increased amounts of connective tissue were seen on the parietal layer of Bowman's capsule.

Alkaline phosphatase enzyme, heavily concentrated in the brush borders of the proximal convoluted tubules and nuclei of normal kidneys, was either absent or sharply reduced in concentration in all damaged areas. Tubules in the regions of increased connective tissue were enzyme-free.

Periodic acid-Schiff (PAS) staining material was observed in Bowman's capsule and in the brush borders of proximal convoluted tubules of normal kidneys. After treatment there was no change in the PAS material of glomerular areas but these mucopolysaccharides disappeared from the proximal tubules. Areas of leucocytes around a tubule were often associated with the presence of PAS-positive material.

The effects on the kidney seen after Su-1621 treatment are reminiscent of those observed in the mouse after testosterone therapy by Van Bekkum and Kassanaar (1951). No consistent influence of Su-1621 was observed on renal sudanophilic lipids.

No pathological changes appeared in the liver following treatment, although there may have been a tendency for an increase in alkaline phosphatase. There were, however, increased numbers of mitoses in the hepatic tissue.

Both the kidney and liver tissue of treated animals had higher water contents than those of normal rats (Table 7). In the case of the liver, and probably the kidneys, the total solids and total proteins of tissues from treated animals were greater than those of of controls (Tables 7 and 8).

Effect of Su-1261 on hepatic regeneration

The observed increase in liver protein despite a reduced appetite and total body weight loss suggested that Su-1621 might be able to facilitate the process of hepatic

regeneration. This was found to be true in experiments conducted as follows: Approximately two-thirds of each liver was removed from a group of female rats. The animals were then divided into two pair-fed groups, one group receiving 4 mg. per day of Su-1621 for 4 days, the other being untreated. On the fifth day autopsies were done. The regenerated livers of treated animals were notably larger than those of controls, a difference apparent on the basis of both wet and dry weights (Table 8).

In further experiments it was found, however, that Su-1621 did not protect either the kidney or liver from the toxic effects of a mercurial diuretic or carbon tetrachloride.

Effect of Su-1621 on nitrogen excretion and nitrogen balance

The observed stimulation of the kidneys and the liver, plus the known ability of testosterone-like steroids to facilitate protein anabolic processes, made it of interest to study the effect of Su-1621 on nitrogen balance. The results of such studies are shown in Table 9, the animals used being the same pair-fed group from which other data are shown in Table 7. Su-1621 did not cause a greater nitrogen retention than that which occurred in pair-fed controls; if anything it reduced the positive nitrogen balance, although no effects of clear significance were established. Both control and treated animals retained less nitrogen after treatment was instituted than before because the aminosteroid, as noted above, caused a reduction in food intake.

The over-all effects of the compound must have involved internal shifts of protein associated with anabolic actions at some sites (e.g. liver) and catabolic changes at others.

Effect of Su-1621 on oxygen consumption

The renotrophic action of certain anterior pituitary extracts is associated with an increased oxygen consumption (White, *et al.*, 1951). For this and other reasons the effect of Su-1621 on the basal oxygen consumption was determined in female rats. The measurements were made by Miss Elizabeth Herrold and her associates in this laboratory, using a Haldane open circuit method adapted to rat work by Dr. Jay Tepperman.

The steroid was given at a dosage of 4 mg. per day for 14 days. Control animals received saline injections and all animals in addition served as their own controls by virtue of determinations made before any treatment began. The results in Table 10 show no effect of Su-1621 at 2 hours or 7 days after treatment began. At 14 days a questionable ($P = 0.05$) increase in oxygen consumption of treated animals was observed when compared with the unusually low values of controls for that day. These data in sum total do

TABLE 9. EFFECT OF Su-1621 ON NITROGEN EXCRETION AND NITROGEN BALANCE IN MATURE PAIR-FED FEMALES

(6 animals per group)

Period observed	Urinary nitrogen, mg./24 hrs./rat		Positive nitrogen balance gm./day/kg.	
	Controls	Treated	Controls	Treated
Pre-treatment	112	92	0.280	0.240
Days 1–4	72	71	0.210	0.140
Days 5–8	52	55	0.120	0.130
Days 9–12	61	78	0.200	0.200
Total during treatment	185	204	0.530	0.470

TABLE 10. EFFECT OF Su-1621 ON OXYGEN CONSUMPTION IN FEMALE RATS

No. rats	Treatment	Oxygen consumption, ml./100 mg./hr.			
		Before treatment	Treated 2 hours	Treated 7 days	Treated 14 days
8	Saline-treated controls	100.1 ±3.4	100.9 ±5.5	104.2 ±4.4	95.4 ±3.6
8	Su-1621, 4 mg. daily	102.2 ±3.3	107.6 ±4.5	96.0 ±5.4	106.9 ±4.1

not establish any true effect of the compound, although they imply that Su-1621 does not alter thyroid function.

Effect of Su-1621 on blood pressure

The fact that Su-1621 caused enlargement of and damage to the kidneys raises the question as to whether it could thereby cause renal hypertension. Therefore, the effect on blood pressure of both acute and chronic treatment was studied. Blood pressure was measured with a photoelectric tensometer (method of Kersten *et al.*, 1947), in animals sedated with sodium pentobarbital (1.8 mg./100 gm.). Such doses of pentobarbital were found not to reduce blood pressure of normal rats below levels obtained without sedation.

Blood pressure was measured at frequent intervals for the first 60 minutes after subcutaneous administration of 4 mg. Su-1621 to 12 adult female rats. In six of these cases blood pressure rose within 30 minutes an average of 19 mm. Hg after which it returned to normal levels within the following 30 minutes. In the other six animals no changes attributable to the steroid were apparent. No reason for the variable responsiveness was detected.

In the chronic experiments, 4 mg./day of Su-1621 were given 10 rats for periods of 30 to 60 days. As is evident from Table 11, no significant change in blood pressure occurred.

Effect of Su-1621 on renal function and water metabolism

The effect of Su-1621 on the excretion of water loads was studied. Five hourly doses of water (3 ml./100 sq. cm. of body surface) were given by stomach tube and the per cent of the water load and amount of chloride excreted one hour after the last water dose indicated in Table 12. The compound had a definite antidiuretic action in females but not males; it did not clearly affect chloride excretion in either sex.

In random observations, not systematized enough to lend themselves to ready tabulation, it was observed that females treated with Su-1621 showed daily water exchanges higher than those of normal animals. This impression plus the more definite results in the

TABLE 11. EFFECT OF PROLONGED TREATMENT WITH Su-1621 ON BLOOD PRESSURE IN FEMALE RATS

(Mean and Ranges of 10 rats, receiving 4 mg/day of Su-1621 for duration of experiment)

Blood pressure, mm. Hg			Terminal organ weights				Body weight change
Before treatment	Treated 10 days	Treated 30–60 days	Kidney, gm./100 gm.	Liver, gm./100 gm.	Adrenal, mg./100 gm.	Thymus, mg./100 gm.	
118 (105–134)	128 (112–148)	115 (105–148)	1.41	5.4	26.3	44	−14%

TABLE 12. EFFECT OF Su-1621 ON WATER AND CHLORIDE EXCRETION AFTER WATER LOADING

(6 rats per group, given 5 hourly doses of water—3 ml/100 sq. cm. body surface—by stomach tube; determinations made on total urine samples, one hour after last dose of water)

Treatment	% water excreted	Chloride excreted		Sex
		mEq./L	Total mEq. ×100	
Controls—untreated	77	9.9	24.25	Female
Su-1621, 4 mg./day ×4—30 min.*	41	20.3	31.30	Female
Controls—untreated	79	9.3	24.25	Female
Su-621, 4 mg./day ×10–30 min.*	37	21.2	28.48	Female
Controls—untreated	79	9.6	22.56	Female
Su-1621, 4 mg./day ×5–24 hours*	52	10.7	12.97	Female
Controls—untreated	74	9.6	25.66	Male
Su-1621, 4 mg./day ×10–30 min.*	75	10.99	29.33	Male

* Indicates time after last injection of Su-1621 that water tests were started.

acute experiments (Table 12) suggest that the large kidneys of Su-1621-treated animals lack the adaptability to conserve water normally under ordinary conditions or to eliminate it normally under conditions of water loading.

Renal clearance tests were then made, using creatinine and paraaminohippurate essentially according to the methods of Boss, Osborn and Renzi (1952). Despite the fact that treated animals had kidneys more than double the size of controls, clearance values were greatly reduced (Table 13). This indicates a reduction in both filtration rate and renal plasma flow and implies again that the renal enlargement is associated with pathological renal dysfunction.

Effects of Su-1092, 20-amino-3β-hydroxy-5-pregnene monohydrochloride

Cursory observations were made on the effects of another aminosteroid, Su-1092. While these were not complete enough for any detailed analysis of its effects, certain interesting comparisons with Su-1621 were suggested. From Table 14 it is clear that Su-1092, unlike Su-1621, had little effect on kidney size. Its hepatotrophic effects were, if anything, greater than those of Su-1621. Su-1092 induced a great enlargement of the spleen, an effect not noted at all with Su-1621. There was a sex difference in response to Su-1092 but it was less complete than that seen with Su-1621. Females only showed a clear-cut effect of the compound on liver weight while both sexes showed an inhibition of body growth and enlargement of the adrenals and spleen.

TABLE 13. EFFECT OF Su-1621 ON RENAL CLEARANCES

(Adult females treated with 4 mg./day Su-1621 for 10 days. Tests made on 11th day. Means and ranges quoted)

Treatment	No. rats	Creatinine clearance, ml./100 gm./min.	Paraaminohippurate clearance, ml./100 gm./min.	Water excretion, ml./min.	Kidney wt.,* gm./100 gm.
Controls	8	1.03 (0.84–1.19)	3.47 (2.2–4.2)	0.11	0.73
Su-1621	8	0.22 (0.11–0.39)	0.66 (0.2–1.2)	0.04	1.76

* Based on 4 animals only for each group.

TABLE 14. EFFECTS OF Su-1092

No. rats	Sex	Treatment	Body weight, gm.			Kidney wt., gm./100 gm.	Liver wt., gm./100 gm.	Adrenal wt., mg./100 gm.	Thymus wt., mg./100 gm.	Spleen wt., mg./100 gm.
			Init.	Term	% Ch.					
7	♀	Controls—untreated	221	235	+ 6	0.79	3.87	25.6	140	237
10	♀	Su-1092, 4 mg./day ×10	221	223	0	0.84	5.49	34.0	74	690
3	♂	Controls—untreated	273	327	+20	0.77	4.52	13.4	148	268
10	♂	Su-1092, 4 mg./day ×10	269	267	0	0.87	4.73	20.9	88	558

DISCUSSION

It is obvious from the observations reported here that the aminosteroid, Su-1621, has some bizarre biological properties. One point of particular interest concerns the basis of the marked sex difference in responsiveness to the compound, a difference apparent by physiological (e.g., diuresis) as well as morphological criteria. The fact that prepuberal castration did not eliminate this sex difference suggests that it is not conditioned entirely by gonadal hormones. The possibility was not ruled out, however, that an early action of the gonads, before the twenty-second day of life, did provide the "cause" for the sex difference. Such a mechanism is clearly possible in view of the long-known fact that post-natal gonads, exerting some influence during the first few days of life, determine a subsequent pattern of pituitary gonadotrophic activity (Pfeiffer, 1940; Bradbury, 1940).

Whether or not some aspect of the sex difference in responsiveness to Su-1621 is independent of gonadal activity, it is true, nevertheless, that the injection of estrogens made non-responsive males or castrates immediately and fully responsive to the compound. This fact makes it highly probable that an action of estrogen contributes to responsiveness in normal females.

The action of testosterone in inhibiting responsiveness, while equally definite under proper conditions, was less immediate and easily demonstrated. Testosterone did not block responsiveness in mature females when it was given simultaneously with Su-1621. It possibly would have done so if it had been given for a considerable period prior to the administration of Su-1621. Testosterone did, however, block responsiveness of the prepuberal castrate female when given for a prolonged period and this fact suggests that the presence of testosterone, as well as the relative absence of estrogen, contributes normally to the refractoriness of the male.

Two questions of immediate interest concerning a steroid such as Su-1621, structurally resembling testosterone but lacking androgenic activity, are the following: (1) Will it block the action of the active sex steroids? (2) Does it retain protein anabolic properties in the absence of androgenic action? The first question is not adequately answered by the data reported here, although some suggestion of a gonad hormone-blocking action might

have been anticipated had it occurred. As to the second question, all of our evidence is consistent with the idea that Su-1621 lacks the general protein anabolic activity of testosterone, when that activity is measured in terms of nitrogen balance. It is barely possible that a true over-all anabolic activity of Su-1621 could be demonstrated with lower and less toxic doses of the compound than those used here. The toxicity of Su-1621 and the reduction of appetite induced by it complicates attempts to study any inherent effects of the compound on protein metabolism by nitrogen balance methods. Perhaps it is significant that despite its toxicity it did not produce negative nitrogen balance. In any event, the compound did have localized anabolic actions as exemplified by (a) the increase in kidney size—the interpretation of which, however, is complicated by the renal pathology involved and (b) the increase in liver mass and the stimulation of hepatic regeneration. In the case of the liver, no evident pathology complicated what seemed to be a true localized anabolic action.

SUMMARY

1. Some of the biological actions of an amino derivative of testosterone, 17-amino-4-androsten-3-one monohydrochloride (Su-1621), were studied in rats.

2. The compound lacked gonad hormone-like activity. In females, it caused renal, hepatic, and adrenal hypertrophy, thymic atrophy, weight loss or diminution in growth rate and reduced appetite. Males rats, on the other hand, showed only slight if any response to the compound.

3. The renal but not the hepatic hypertrophy was associated with a marked pathology. The enlargement of these organs was in part due to hydration but in part to protein deposition as well.

4. Su-1621 caused lesions at subcutaneous injection sites but it could be given without difficulty and without apparent loss of activity by stomach tube. It caused hemolysis of dog erythocytes *in vitro*. Acute overdosage caused convulsions and death without evidence of steroid anesthesia.

5. In pair-fed animals no protein anabolic effects of Su-1621 could be demonstrated by nitrogen balance studies. The fact that it caused hepatic hypertrophy and a stimulation of hepatic regeneration, however, indicated a "trophic" action on that organ.

6. Both creatinine and paraaminohippurate clearances were greatly depressed by treatment with large doses of Su-1621, indicating that the characteristic renal hypertrophy was associated with renal dysfunction. The compound also induced abnormalities in water metabolism.

7. Castration did not result in responsiveness to Su-1621 in males nor abolish it in females. Estrogen administration, however, caused immediate

responsiveness in males whether given with or preceding Su-1621. Testosterone propionate did not block responsiveness when given with Su-1621 in mature females but did so when given for 39 days after pre-puberal ovariectomy. Gonadal hormones are obviously conditioning factors in the response to Su-1621 but it is not established that they are the sole determinants of the sex difference in responsiveness.

8. A related steroid, Su-1092, 20-amino-3β-hydroxy-5-pregnene monohydrochloride, had properties resembling in part but not entirely those of Su-1621.

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THE MAMMARY GLAND SPREADING FACTOR IN NORMAL PREGNANT ANIMALS[1]

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IN A number of species it has been shown that growth of the mammary gland occurs chiefly during the first half to two-thirds of pregnancy (Turner 1939 a and b, Folley, 1952). The duct system first and later the lobule-alveolar system penetrates into a fatty pad of connective tissue. It was suggested (Elliott and Turner, 1950) that the forward progress of the growing mammary gland would be facilitated if the cells of the end-buds of the ducts, and later the growing side-branches which form the alveoli, produced a spreading factor which acted to break down some component of the connective tissue, possibly the ground substance.

A preliminary investigation (Elliott and Turner, 1950), showed that such a spreading factor was present in the mammary glands of pregnant rats and could be extracted and assayed. In the present report, the investigation has been extended to determine if a mammary gland spreading factor such as was found present in the rat glands was present in other species of experimental animals during pregnancy. In addition these experiments were designed to help determine whether or not the pattern of increase in amounts of mammary gland spreading factor paralleled the pattern of growth in the mammary gland during pregnancy, thus indicating that the spreading factor may play a role in the extension of the growing mammary gland into the fatty pad.

METHODS AND MATERIALS

In this study mature nulliparous females of each species were employed. Standard methods of inducing and determining pregnancy were utilized. The mammary glands were removed, extracts made and the amount of spreading factor in the extracts determined by a method previously described (Elliott and Turner, 1950). However, the weights of the mammary glands of the various species varied greatly and since it was desirable to have assays on the various species comparable; the amount of buffer used to extract the mammary glands was figured on a per weight basis. The ratio of 6.25 ml. for 1 gm. mammary tissue was employed. Since the weight of the rat mammary

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gland was about 4 gm., 25 ml. was used. The normal mouse was found to have a mammary gland of about 0.8 gm., so 5 ml. of buffer was employed. The guinea pig had a mammary gland which averaged 4.5 gm. and 30 ml. of buffer was used. The mammary glands of the normal female rabbit varied greatly. However, the average weight appeared to be about 28 gm. and 175 ml. of buffer was employed.

RESULTS AND CONCLUSIONS

The Albino Rat. The preliminary report (Elliott and Turner, 1950) showed that the mammary glands from non-pregnant animals contained no spreading factor, while the mammary glands taken from a large number of rats at various stages of pregnancy showed measurable amounts of spreading factor except during the first and last two days. The amount of spreading factor increased with each day of pregnancy until a maximum was reached on about the twelfth day, following which there was a decline in the amount of spreading factor during the remainder of pregnancy.

The growth of the mammary gland during pregnancy is considered to be largely completed in from 12 to 14 days in the rat. Following this period the amount of cellular division slows down greatly and milk secretion is initiated. As indicated by these data, the amount of spreading factor in the mammary gland increased until it reached a peak near the time when growth is considered to be largely completed after which the amount of spreading factor decreased. Since the number of animals used in this investigation was by far the largest for a single species, the results show clearly the rise and decline in the amount of spreading factor at the various stages of pregnancy (Fig. 1).

The Albino Mouse. Assays of the mammary glands from normal female mice indicated that no spreading factor was present in the non-pregnant animals. The first evidence of the presence of the spreading factor was observed at the fourth day of pregnancy. At this time the mammary glands contained some spreading factor. The amounts of spreading factor gradually increased with advancing pregnancy until a peak was reached on the twelfth day after which it gradually declined. No spreading factor was detectable in the mammary gland extracts of mice pregnant 19, 20 or 21 days.

The period of growth of the mammary gland during the gestation period is approximately the same for the mouse and rat. Therefore, it is not surprising to find that the amounts of spreading factor in mice and rats parallel each other during the various stages of pregnancy.

While fewer mice were employed in this investigation in comparison to the number of rats studied, the curve showing the amounts of spreading factor in the mammary glands of mice (Fig. 2) during pregnancy follows the same pattern as that shown by the rats.

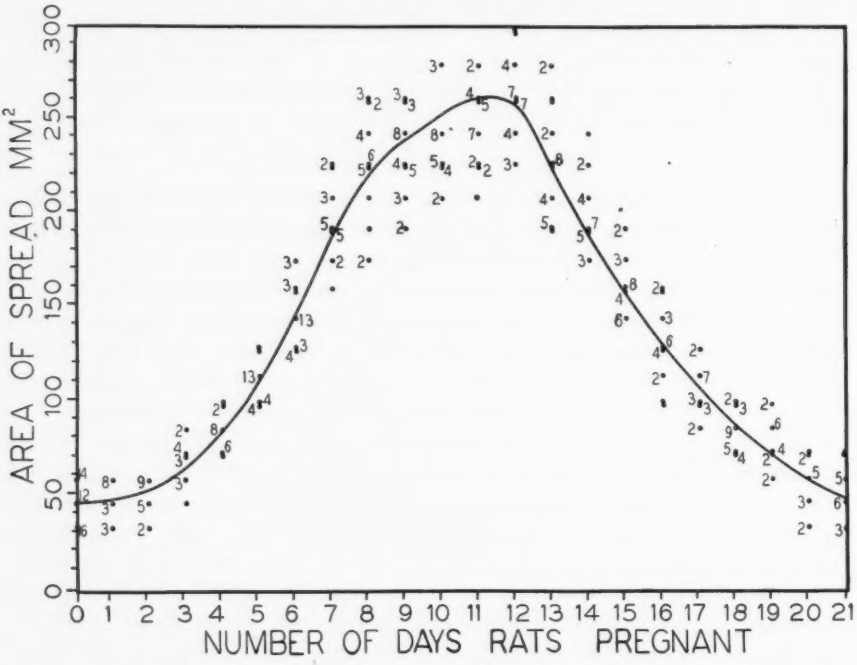

FIG. 1. It will be observed that the amount of mammary gland spreading factor in the rat increases during pregnancy reaching a peak on the 12th day. The rise in amount of spreading factor parallels the growth phase of mammary gland growth. Following the peak, the amount of spreading factor declines rapidly as gland growth declines and secretory activity is initiated.

The Guinea Pig. As in the studies with the two previous species, the mammary glands from non-pregnant guinea pigs showed no spreading factor. Mammary glands from guinea pigs pregnant 5 and 7 days also showed no spreading factor. Extracts of glands from animals pregnant 10, 13 and 15 days indicated the presence of small amounts of the spreading factor. During the rest of early pregnancy there was an increase in the amount of spreading factor in the mammary glands until a peak was reached on about the 41st day, after which there was a decline. By the 60th day of pregnancy very little spreading factor could be detected in the mammary gland and on the 62nd day the assays showed none. The mammary glands from two guinea pigs at parturition, pregnant 66 and 67 days showed that no spreading factor was present.

Here again the amount of spreading factor parallels the growth of the mammary gland during pregnancy (Fig. 3). As in the case of growth,

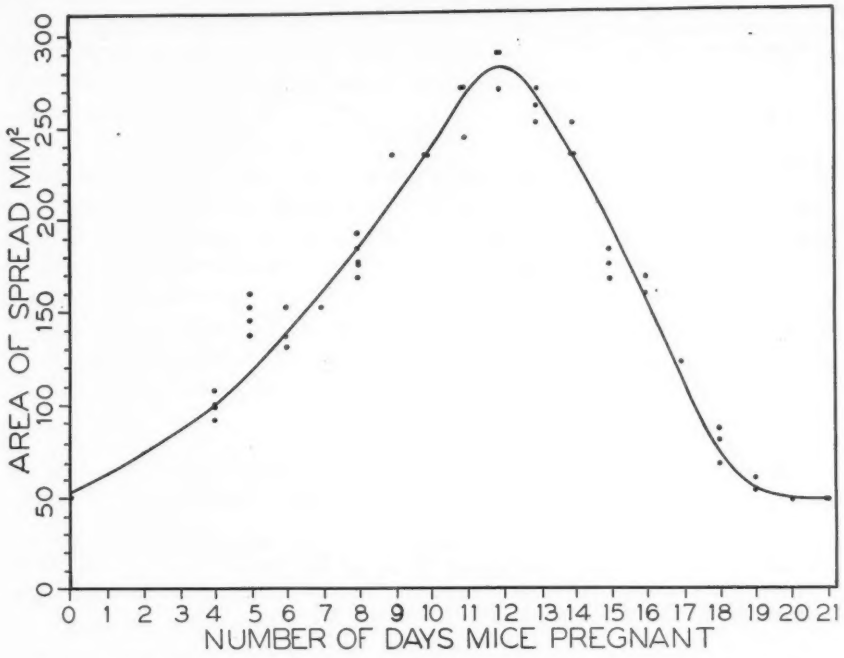

FIG. 2. The amount of mammary gland spreading factor present in the glands of pregnant mice increased to 12 days similar to that of the rats during the growth phase. There was a rapid decline in spreading factor as milk secretion was initiated.

very little spreading factor activity is present before the 15th day, after which both increase gradually. The peak amount of spreading factor was observed on the 41st day of pregnancy. While the growth period of the mammary gland is not known exactly, the two-thirds point of pregnancy, probably the time of maximum growth, would fall between 40 and 45 days. Thus, the maximum spreading factor activity and maximum mammary gland growth occurred at about the same time in the guinea pig. The decline in amounts of the spreading factor after the 41st day also agrees with the decrease in numbers of mitotic figures known to occur in the mammary gland during this period of pregnancy.

The Rabbit. Assays of the mammary glands of non-pregnant rabbits showed that no active spreading factor was present. The mammary glands from the earliest stage, at 4 days of pregnancy, showed little or no spreading factor was present. The amounts of mammary gland spreading factor increased until a peak was reached on the 18th day of pregnancy after which there was a gradual decline. By the 28th day of pregnancy no spreading factor was detectable in the mammary gland extracts.

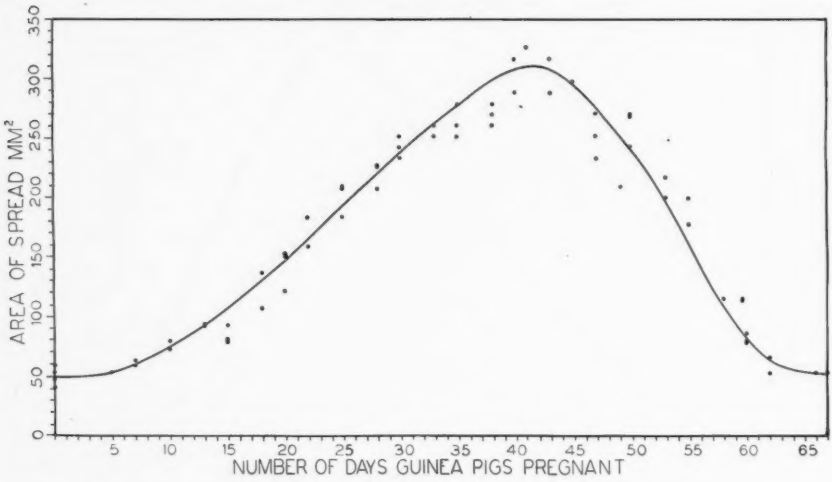

FIG. 3. In the guinea pig, with a long period of pregnancy, the rise in the amount of spreading factor present in the mammary glands was gradual up to the peak at 40 to 45 days. The subsequent decline in spreading factor with the cessation of growth and the initiation of lactation was quite rapid.

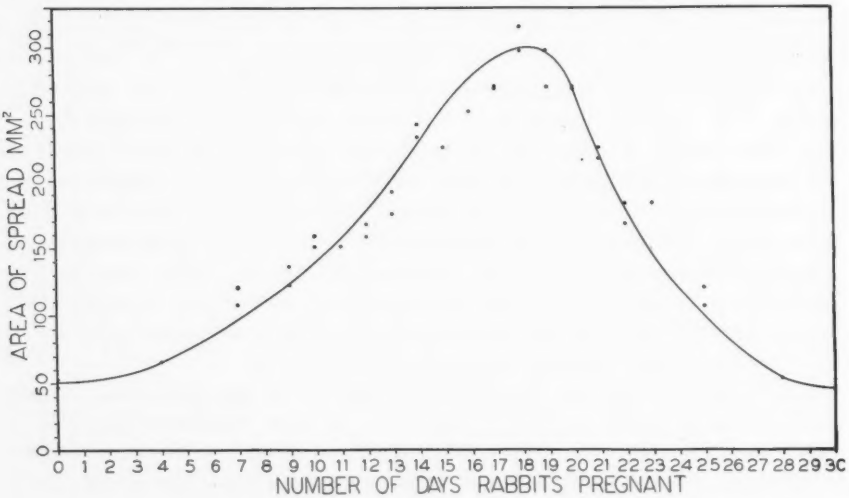

FIG. 4. The rise and decline in the amount of mammary gland spreading factor in the pregnant rabbit in relation to growth and the initiation of milk secretion follows the pattern of the other species.

Again the increase in amounts, a peak at the 18th day and a decline in amounts of spreading factor during pregnancy (Fig. 4) checks with the pattern of growth occurring in the pregnant rabbit.

SUMMARY

Investigations in rats, mice, guinea pigs and rabbits have shown that the increases and the periods at which peak amounts of the spreading factor were observed to be present in the mammary glands during pregnancy closely parallels the growth phase of mammary gland development, reaching a maximum between mid and $\frac{2}{3}$ of gestation. During the last $\frac{1}{3}$ of pregnancy, when cells of the mammary gland begin secretion, amounts of the spreading factor decline. It would appear that elaboration or activation of the spreading factor practically ceases as secretory activity begins.

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SOME METABOLIC STUDIES ON HYPOPHYSECTOMIZED PIGS

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METABOLIC studies have been reported on several species of hypophysectomized animals including the monkey, dog, cat, rabbit, rat and toad. Hypophysectomized animals readily become hypoglycemic and may present grave symptoms, frequently terminating in death. Treatment with sugar produces improvement but must be initiated early and repeated frequently (Houssay, 1937). Hypophysectomy alters nitrogen balance and retention of nitrogen appears to be dependent on an anterior lobe factor (Lee and Ayres, 1936).

The following experiment was conducted to obtain information on some metabolic processes in the hypophysectomized pig. Of special interest was the effect of fasting on blood glucose, non-protein nitrogen and uric acid.

EXPERIMENTAL

Pigs of mixed breeds were hypophysectomized by the temporal approach when approximately eight weeks of age. Most of those that did not eat voluntarily following surgery were nourished by gavage. When they recovered and resumed eating and drinking, a balanced ration (containing 16% protein) and water were allowed ad libitum. All pigs were kept in warm quarters. The pigs were subjected to a 48-hour fast ten weeks after hypophysectomy. Control pigs of similar age were fasted 120 hours to study the effect of a more prolonged fast.

The blood studies were divided into two phases: (1) the ten-day period immediately following hypophysectomy and (2) during a 48-hour fasting period ten weeks after hypophysectomy. During the first phase blood was collected before the operation and at 24-hour intervals thereafter. Blood samples were collected at the beginning and at 12-hour intervals during the fast. Seven normal pigs of similar age and fed the same ration were used as controls.

Blood samples were collected from the anterior vena cava according to the technic described by Carle and Dewhirst (1942). Sodium fluoride

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was used as anticoagulant. The following methods were used in the determinations on the blood: total non-protein nitrogen, Koch and McMeekin (1924), uric acid, Folin (1930), blood glucose, Somogyi (1945).

At the termination of the study the animals were autopsied. Endocrine tissues were collected for further study. Complete removal of the hypophysis was affirmed by histological examination of any tissue in the pituitary fossa. Unless otherwise indicated the data included in this report are from twelve animals found to have complete removal of the hypophysis. Data were also obtained on 7 pigs which had incomplete removal of the hypophysis.

RESULTS

It was necessary to feed most of the pigs by gavage for several days immediately following hypophysectomy. Rapid, fatal hypoglycemia developed if this was not done. Two pigs which ate very little and were not fed by gavage had blood glucose values which averaged 11.6 mg./100 ml. when observed in a comatose condition 4 days after hypophysectomy. Intraperitoneal administration of glucose solution did not save the lives of these animals suggesting that the hypoglycemia rapidly approaches an irreversible state. The average pre-operative glucose value for these 2 pigs was 60.1 mg./100 ml. (Fig. 1).

Blood glucose and non-protein nitrogen values obtained from adequately nourished hypophysectomized pigs during a 10-day post-operative period closely paralleled those from normal pigs of similar age. Values for all the pigs were within the normal range for animals of that age (Fig. 1).

There was a rapid fall in blood glucose when the hypophysectomized pigs were fasted 48 hours. The average prefasting concentration of 64.4 mg. was reduced to 37.2 mg., a decline of 27.2 mg./100 ml. within 48 hours. The blood non-protein nitrogen concentration decreased from 37.7 to 35.1 mg., an average of 2.6 mg./100 ml. and the uric acid from 0.98 to 0.13 mg./100 ml. during the fasting period. One pig, found comatose after a 36-hour fast had a blood glucose value of 15.5 mg./100 ml.

There was little change in the blood glucose in the fasted normal pigs even at the end of 120 hours. However, the blood non-protein nitrogen increased an average of 6.42 mg. and the uric acid 2.9 mg./100 ml. within 48 hours. There was greater increase in both of these constituents at the end of 120 hours (Fig. 2).

A small amount of pituitary tissue appears sufficient to maintain normal blood glucose and non-protein nitrogen values in fasted pigs. Pigs which possessed 10% or more of the anterior pituitary tissue maintain blood glucose and non-protein nitrogen values comparable to fasted normal pigs (Fig. 3).

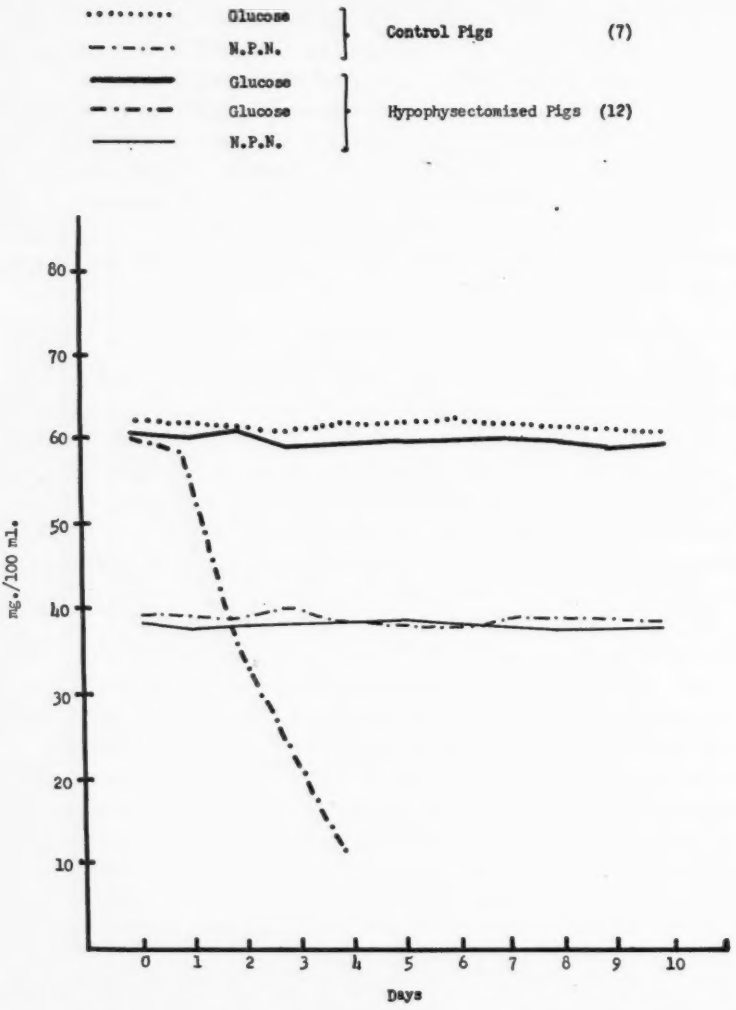

FIG. 1. Blood glucose and non-protein nitrogen concentrations from normal and hypophysectomized pigs for ten-day period after surgery. Hypophysectomized pigs which did not eat voluntarily were nourished by gavage except two indicated by —·—·— line. Figures in parentheses indicate number of pigs in each group.

DISCUSSION

Investigators have found several species of animals to be subject to rapid fatal hypoglycemia immediately following hypophysectomy unless preventive measures are taken. This may be due mainly to failure to consume

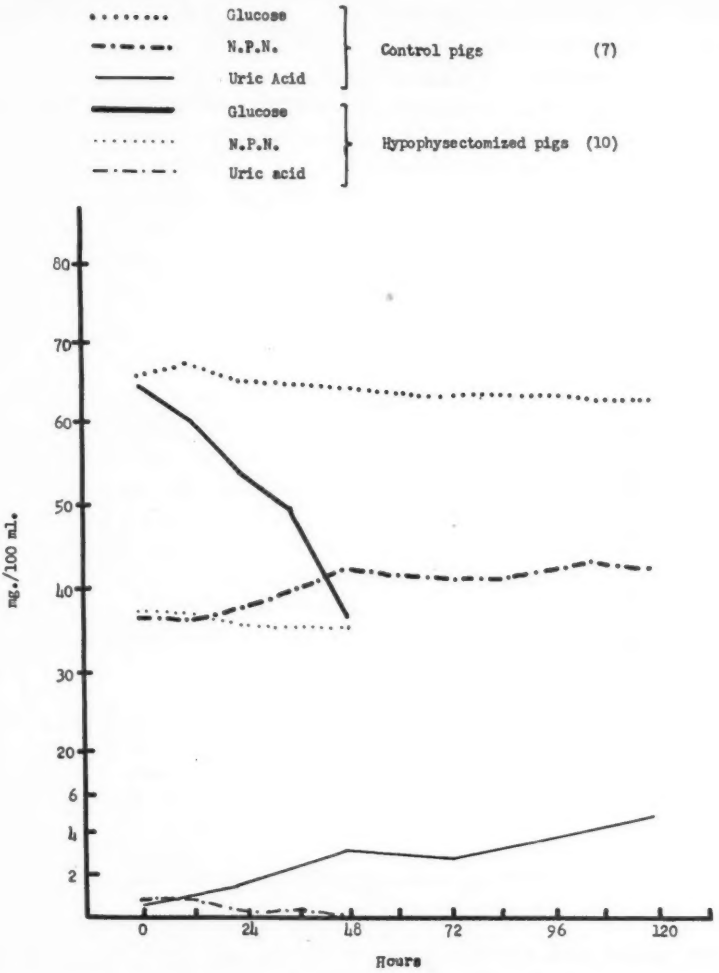

FIG. 2. Blood glucose, non-protein nitrogen and uric acid concentrations from fasting normal and hypophysectomized pigs. Pigs were of similar age. Figures in parentheses indicate number of pigs in each group.

food during this period because force feeding or the parenteral administration of nutrients usually prevents the post-operative hypoglycemia.

The rapid decline in the blood glucose when hypophysectomized pigs are fasted is similar to that observed in other species. Bennett (1936) and Russell (1936), found that hypophysectomized rats absorbed carbohydrate at a slower rate and stored less glycogen in the tissues than normal rats.

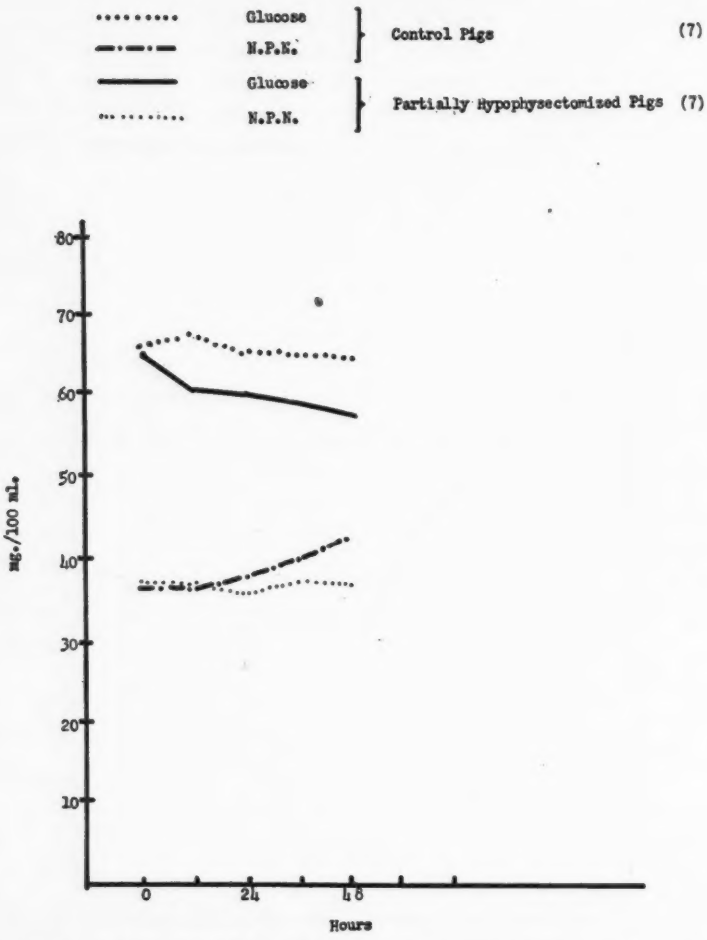

FIG. 3. Blood glucose and non-protein nitrogen concentrations from normal and partially hypophysectomized pigs during a 48-hour fast.

Schaffer and Lee (1935), found less nitrogen in the bodies of hypophysectomized animals than in their pair-fed controls. The lowered reserve of readily available carbohydrate and nitrogen plus a probable reduction in gluconeogenetic activity may contribute to the rapid onset of hypoglycemia. There was, however, little variation in the blood glucose of the hypophysectomized and control pigs at the beginning of the fast. If the hypophysectomized pig absorbs glucose at a slower rate, it is not reflected in a lower blood concentration when the animal is adequately nourished.

The reduction in total blood non-protein nitrogen and uric acid in fasted hypophysectomized pigs suggests that endogenous protein metabolism is affected by removal of the pituitary gland.

Data obtained from fasting humans indicate that there may or may not be an increase in the blood non-protein nitrogen (Lennox *et al.* 1926). The concentration of non-protein nitrogen in the blood has been used clinically as an indication of kidney function (Keys *et al.*, 1950). However, blood uric acid shows a consistent increase during acute starvation (Hoeffel and Moriarty, 1924, Lennox, 1925). Results from this study indicate that blood uric acid increases in fasted normal pigs whereas it decreases in hypophysectomized pigs.

SUMMARY

The fasted hypophysectomized pig develops a rapid, fatal hypoglycemia. There is also a decrease in blood uric acid and total non-protein nitrogen. In the fasted normal pig there is only a slight reduction in blood glucose, but there is an increase in non-protein nitrogen and uric acid. The rapid onset of hypoglycemia and the lowered blood uric acid in fasted hypophysectomized pigs indicate that there is a low glycogen reserve in the liver and a decrease in gluconeogenic activity. The blood glucose and non-protein nitrogen values of fasted partially hypophysectomized pigs paralleled those of fasted normal pigs.

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STUDIES IN SPERM HORMONES: DEMONSTRATION OF ESTROGENIC ACTIVITY

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THE mechanism by which the testes exert a regulatory influence on the anterior pituitary is still an obscure physiologic phenomenon. While large doses of testosterone may depress the pituitary gonadotrophins, it is now generally agreed that physiologic quantities of this hormone do not inhibit the secretion of gonadotrophin to a significant extent. This would indicate that, either there are other testicular hormones capable of inhibiting gonadotrophins, or that some other mechanism of pituitary control is involved. The first of these hypotheses received some measure of support as it became evident that the testis is capable of producing estrogenic hormones. Such evidence came from the isolation of estrogens from the testes of the stallion (Beall, 1940), hog (Ruzicka, 1943; Haines *et al.*, 1948), and more recently from human testes by Goldzieher and Roberts (1952). Since estrogens are known to be powerful pituitary inhibitors, these isolation experiments add support to the view that these hormones may play a role in the control of anterior pituitary gonadotrophin activity. For reasons that will be examined in this article, we believe that the tubular epithelium plays a primary role in this mechanism of pituitary control. It is therefore of interest to report on the occurrence of estrogenic substances in the sperm, which for practical purposes may be considered a part of the seminiferous epithelium. Whether or not there is sufficient estrogen represented here to produce physiologic pituitary inhibition has not been established. This question has been the subject of preliminary reports (McCullagh and Schaffenburg, 1951, 1952). In the present communication we wish to report on further studies with fractionated extracts of sperm.

METHODS AND RESULTS

Whole bull semen was obtained in the frozen state from agricultural experiment stations[2] and stored in a deep freeze unit until adequate quantities had been accumulated

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[1] Material submitted as part of the requirements for the degree of M.S.

[2] We wish to express our thanks to Dr. F. X. Gassner, Colorado Agricultural Experiment Station, Fort Collins, Colorado, and to Mr. G. W. Thompson, Western Pennsylvania Artificial Breeding Cooperative, Clarion, Pennsylvania, for the generous donation of bull semen used in these studies.

for extraction. After allowing the semen to thaw at room temperature, the plasma was separated by centrifugation and discarded. The packed cells were then washed twice with physiologic saline and again separated by centrifugation. Distilled water in amounts sufficient to insure a homogenous suspension of the cells was added and the homogenate again placed in the deep freeze. Thawing was allowed to occur at room temperature in order to obtain lysis of the sperm. The aqueous suspension was hydrolyzed for 20 minutes at 20 lbs. in a pressure cooker after addition of 10 volumes % concentrated HCL. Extraction was carried out three times by addition of alcohol to 70% concentration. Alcohol was removed by distillation under reduced pressure, and the aqueous material extracted three times with 20 volumes % of ethyl ether. With some extracts the packed cells were also directly extracted with ethyl ether and the ether fractions pooled. Fractionation of the ether extract into neutral and crude phenolic fractions was then carried out according to the method of Friedgood and Garst (1948). Extract number 137/52 yielded the following quantities of material from 590 ml. of whole semen:

Crude neutral fraction	353.5 mg.
Crude phenolic fraction	252.7 mg.

Assuming an average sperm count of 1.3 billion cells per ml. (computed from averages of previous batches of semen assayed in our laboratory) this would indicate a yield of approximately 1.0 mg. per 3.04 billion cells for the phenolic fraction and per 2.17 billion cells for the neutral fraction. Extracts, after being taken to dryness, were dissolved in peanut or cotton-seed oil in amounts convenient for injection.

The crude phenolic fraction of 137/52 was assayed in two groups of eight animals each of spayed, adult albino rats, weighing on an average 120 gm. Two-tenths ml. of extract were given subcutaneously daily for 4 days in doses totalling 1.0 mg./rat for group 1, and 0.5 mg./rat for group 2; spayed, uninjected animals served as controls. Vaginal smears were taken daily and the animals sacrificed on the fifth day. Uteri were fixed in Zenker's solution and weighed. Results are presented in Table 1. Both dose levels produced full vaginal cornification and increase in uterine weight. That the effect

TABLE 1. EFFECT OF CRUDE PHENOLIC FRACTION OF BULL SPERM EXTRACTS IN THE ADULT, SPAYED RAT

Treatment	Uterus (mg.)	Thymus (mg.)	Vag. smears
1.0 mg. extract	164.2±10.9	278.3±23.9	100% cornif.
0.5 mg. extract	166.5±8.6	349.6±13.56	100% cornif.
Untreated controls	46.6±3.36	378.2±41.8	Negative

was probably maximal at the 0.5 mg. dose level is indicated by the fact that twice that dose did not produce further uterine stimulation. The involution of the thymus observed in the injected animals, although not pronounced, is entirely compatible with the estrogenic effect.

Fractionation of crude phenolic fraction. 104 mg. of crude phenolic fraction of extract 137/52 was further partitioned[3] into purified phenolic, neutral and acidic fractions according to the method of Engel (1950). Yields were as follows:

Purified phenolic fraction	14.3 mg.
Neutral fraction	13.5 mg.
Acidic fraction	30.6 mg.

[3] Carried out in Dr. L. L. Engel's laboratory, Harvard University, Boston, Massachusetts. Further studies on these fractions will be the subject of a future report.

TABLE 2. EFFECT OF FRACTIONS RESULTING FROM THE PARTITION OF CRUDE PHENOLIC FRACTION OF BULL SPERM EXTRACTS IN THE ADULT, SPAYED RAT

Treatment	Uterus (mg.)	Vag. smears
Purified phenolic fraction, 0.5 mg.	72.2±6.32	Negative
Neutral fraction, 0.5 mg.	70.1±2.38	Negative
Acidic fraction, 0.5 mg.	59.2±3.36*	Negative
Untreated controls	74.0±3.58	Negative

* P 0.02 compared to untreated controls.

These fractions were assayed as before at doses of 0.5 mg. total per rat. Results are presented in Table 2, where it will be noted that none of the fractions showed estrogenic activity as judged by either uterine weight or vaginal smears. The fact that the acidic fraction caused an apparent decrease in uterine weight compared to control values (results which were statistically significant), remains unexplained and has not been reproduced in later experiments.

Since it appeared improbable that destruction of active substances had taken place during extraction procedures, and as the question of an estrogen synergist or augmenter, possibly present in the crude phenolic fraction, arose, the 3 fractions mentioned before were re-assayed, singly and in combination, using the method previously described. Doses for the single substances totalled 1.0 mg./rat and 0.5 mg./rat for the combination of the 3 substances, which were mixed in the following proportions according to the amounts originally obtained from the crude phenolic fraction:

Purified phenolic fraction	24%[4]
Neutral fraction	23%
Acidic fraction	52%

Results are presented in Table 3. While the acidic and neutral fractions proved inactive, the purified phenolic fraction was positive by both vaginal smears and uterine weight. As regards the combined fractions, although there was an increase in uterine weight at doses otherwise inactive when each fraction was assayed alone, vaginal smears were

TABLE 3. EFFECT OF PURIFIED PHENOLIC, NEUTRAL AND ACIDIC FRACTIONS OF BULL SPERM EXTRACT, ALONE AND IN COMBINATION, IN THE ADULT, SPAYED RAT

Treatment	Uterus (mg.)	Vag. smears
Purified phenolic fraction, 1.0 mg.	102.8±9.64	100% cornif.
Neutral fraction, 1.0 mg.	77.4±7.31	Negative
Acidic fraction, 1.0 mg.	77.2±4.76	Negative
Recombined above fractions, 0.5 mg.	98.6±9.0	Negative
Untreated controls	79.5±4.12	Negative

negative. The increase in uterine weight caused by this combination of fractions was, however, only on the borderline of statistical significance (P 0.1).

The possibility of an estrogen synergist present in sperm was also apparent in earlier experiments, where saline-washed bull spermatozoa produced a synergistic effect when injected together with estradiol-17β. These results are presented in table 4. Though uterine weight was increased, vaginal smears were negative.

Fractionation and assay of crude neutral fraction. 353.5 mg. of neutral fraction 137/52

[4] More recent analyses have indicated different proportions.

TABLE 4. EFFECT OF BULL SPERM SUSPENSIONS, ALONE AND IN COMBINATION WITH ESTRADIOL-17β, IN THE ADULT, SPAYED RAT

Treatment	Uterus (mg.)	Vag. smears
Saline sperm suspensions	77.5±5.28	Negative
Sperm suspensions plus estradiol-17β, 0.05 μg.	98.0±6.23	Negative
Estradiol-17β, 0.3 μg.	79.0±8.47	Negative
Untreated controls	73.8±3.20	Negative

were separated[5] into ketones and non-ketones by means of Girard reagent T. Further separation into alpha and beta compounds by means of digitonin precipitation was carried out. The following yields were obtained:

Beta ketones	16.5 mg.
Beta non-ketones	70.0 mg.
Alpha ketones	61.9 mg.
Alpha non-ketones	134.5 mg.

These substances were dissolved in cotton-seed oil in amounts convenient for injection and assayed in immature, albino castrate male rats. Each substance was injected subcutaneously daily for 4 days in 0.2 ml. oil in doses totalling 6 mg. per rat. A crude phenolic fraction (138/52) was also assayed by this method, at doses totalling 22.5 mg./rat. Castrate, uninjected male rats served as controls. The animals were sacrificed on the fifth day, sex accessories dissected out and weighed fresh on a torsion balance. Prostatic alkaline phosphatase was determined in tissue homogenates by the method of Huggins and Talalay (1945). Results are presented in Table 5. It will be noted that while all fractions caused an increase in seminal vesicle and prostate weights there was no change in the concentration of alkaline phosphatase in the prostate.

TABLE 5. EFFECT OF CRUDE PHENOLIC FRACTION, α AND β KETONES AND NON-KETONES OF NEUTRAL FRACTION OF BULL SPERM EXTRACTS ON THE SEX ACCESSORIES OF THE CASTRATE, IMMATURE MALE RAT

Treatment		Sem. Ves. mg.	Prostate		
			Weight mg.	Alk. phosphatase	
				Units/gland	Units/100 mg.
β Ketones	3 mg.	8.6±0.81	25.6±1.52	1.56	6.12
β Non-ketones	3 mg.	7.8±0.60	25.4±1.68	1.59	6.24
α Ketones	3 mg.	10.9±0.53	26.3±1.32	1.76	6.63
α Non-ketones	3 mg.	8.8±0.72	27.9±2.45	1.72	6.32
Crude phenolic fraction, 22.5 mg.		7.0±0.86	25.0±1.65	1.59	6.32
Untreated controls		5.3±0.49	17.5±0.60	1.10	6.26

DISCUSSION

The demonstration of estrogenic substances in sperm seems to clarify some hitherto unexplained observations in testicular physiology. It has been repeatedly noted that tubular damage leads to excess gonadotrophin

[5] We wish to express our appreciation to Dr. Kenneth Savard of the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, for his advice in the preparation of these extracts.

production. Evidence for this has been recently reviewed (McCullagh and Schaffenburg, 1952). It has been assumed that the tubular epithelium produces pituitary-inhibiting substances, possilby estrogenic in nature. Disagreement exists, however, both as regards the mechanism of action involved and as to the source of the estrogenic substances in the testes.

Heller *et al.* (1952) have proposed the hypothesis that the pituitary gonadotrophins are utilized by the seminiferous tubules and that when damage to these structures occurs the nonutilized gonadotrophins are eliminated in excessive amounts in the urine. Actual proof for the gonadal utilization of pituitary hormones is, at present, still lacking, and, besides, while this proposed mechanism might well be operative, at least in part, it would be difficult to understand, solely on its basis, the pituitary changes occurring after castration or the menopause (Biggart, 1934, 1935; Witschi, 1940). Here, in the functional or anatomical absence of the gonads, there are castration changes in the pituitary or actual increase of its gonadotrophin content. It seems likely, therefore, that in the absence of gonadal tissue there must be overproduction of pituitary gonadotrophins as well as lack of utilization at the target organs.

Inhibition of the pituitary by the testes has been considered; however, lack of testosterone alone, as was pointed out before, cannot explain the increase in pituitary gonadotrophin.

It has been postulated that estrogen may be the testicular factor producing pituitary inhibition (Howard *et al.*, 1950). Its source within the testis, however, is still debated. While Huggins and Moulder (1945), Berthrong *et al.* (1949) and del Castillo *et al.* (1947) have placed it in the Sertoli cells, Maddock *et al.* (1952) believe it comes from the Leydig cells. The evidence for the former lies in the fact that Sertoli cell tumors in the dog (Huggins and Moulder, 1945; Berthrong *et al.*, 1949) and presumably in the human (Teilum, 1949) are capable of causing feminization. Berthrong *et al.* (1949) isolated large amounts of estrogen from the testis of one dog suffering from a Sertoli cell tumor. Maddock *et al.* (1952) base their evidence for the Leydig cell origin of testicular estrogens on the fact that administration of chorionic gonadotrophin to men with intact testes causes a pronounced increase in the urinary excretion of estrogen, while this does not occur in men suffering from testicular failure. The increased excretion of estrogen is accompanied by evidence of Leydig cell stimulation while the tubules remain atrophic.

In the present communication, evidence is adduced for the presence of estrogenic hormones in the sperm. Since the sperm may be considered a part of the seminiferous epithelium it is probable that these hormones are formed in these structures. The possibility remains that hormonal sub-

stances formed in the interstitial tissue cross the basement membrane and are carried along with the sperm. The fact that the active extracts were prepared from washed cells, however, makes this possibility unlikely. Injections of whole semen, or plasma alone have proved extremely toxic[6] to the experimental animals, and therefore, these experiments have been inconclusive. The study of extracts prepared from semen plasma is being contemplated.

From the experiments presented it seems probable that besides the estrogenic hormones present in the purified phenolic fraction, other substances with augmenting or synergistic properties, are also present in the crude phenolic material. This seems indicated by the fact that smaller doses of the crude material than of the purified phenolic fraction are capable of inducing estrus and by the synergism of natural estrogens observed with the sperm suspensions. Indeed, this line of thought would be in agreement with the recent findings of Cohen and Bates (1952) who have reported the presence of an augmenter in phenolic extracts of mare's urine.

As regards the crude neutral fraction of our extracts, it appears that no active androgenic activity is present as indicated by the lack of prostatic alkaline phosphatase stimulation, in spite of a small increase in the weight of this organ. This is in contrast to a previous communication (McCullagh and Schaffenburg, 1951) in which some phosphatase stimulation was caused by the injection of neutral fraction from human sperm. It would also be in contrast to the reports of Dirscherl and Kmüchel (1950), who reported that sperm extracts give a positive color reaction which they believe to be characteristic of substances of the trans-dehydroandrosterone group. In unpublished observations from this laboratory we have been unable to obtain a positive Zimmermann reaction with some of our neutral fraction preparations. It appears possible that the increase in seminal vesicle and prostate weight obtained in our experiments was due to contamination of the neutral fraction with phenolic material. Indeed, Friedgood and Garst (1948) have reported that up to 26% of the phenolic material may be left behind in the ether fraction when this is extracted with 2 N NaOH.

So far, the demonstration of estrogens in sperm has only been accomplished with material obtained from bulls. Although our preliminary experiments with human sperm suspensions and small quantities of extracts have indicated that similar activity may be expected this remains for future studies to demonstrate.

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[6] The toxicity formerly reported in some of the extracts (McCullagh and Schaffenburg, 1951) was perhaps due to the presence of spermine (Rosenthal *et al.*, 1952).

SUMMARY

Estrogenic activity has been demonstrated in extracts prepared from bull sperm, which caused vaginal cornification and increase in uterine weight in the spayed, adult albino rat. The possible presence in these extracts of an augmenter or synergist of estrogenic action, similar to that described by Cohen and Bates (1952) for mare's urine, is suggested by the increased activity demonstrated by crude phenolic fractions as compared to that of purified phenolic material.

No androgenic activity was demonstrated in the neutral fraction of extracts fractionated into α- and β-ketones and non-ketones, as judged by the lack of increase in the prostatic alkaline phosphatase of the immature, castrate male rat.

The presence of estrogens in sperm, which may be considered a part of the testicular tubular epithelium, may explain some hitherto obscure phenomena in the control of the anterior pituitary by the testes.

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THE ACTIVITY OF EXTRACTED PANCREATIC HYPERGLYCEMIC-GLYCOGENOLYTIC FACTOR AFTER COBALTOUS CHLORIDE AND SYNTHALIN A

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THERE is much evidence that the alpha cells of the pancreatic islet tissue are responsible for a biologically active secretion (Heard, Lozinski, Stewart and Stewart, 1948; Sutherland and deDuve, 1948; Sutherland, Cori, Haynes and Olsen, 1949). Its extraction, purification and autonomy have been described (Audy and Kerly, 1952; Stewart, 1948; Sutherland and deDuve, 1948; Zimmerman and Donovan, 1948); though extracted along with insulin, it is chemically, physically and biologically distinct from that molecule (Pincus, 1950; Sutherland, Cori, Haynes and Olsen, 1949). Because of two most apparent biological properties, namely the release of glycogen as glucose from liver slices and hyperglycemia from intravenous or intramuscular injection, it has been named the hyperglycemic-glycogenolytic factor, or HGF. A hypothetical third effect, an influence on ketone metabolism, (Thorogood and Zimmerman, 1945; Fodden 1953b) requires more investigation.

Two chemical compounds, cobaltous chloride and synthalin A (decamethylenediguanidine hydrochloride) have been used to injure or ablate the alpha cells in the living animal, and to study the effects of their withdrawal upon glucose metabolism (Davis, 1952; Fodden, 1953b; Goldner, Volk and Lazarus, 1952; Van Campenhout and Cornelis, 1951). The results have been paradoxical and confusing. Cobaltous chloride appears to cause extreme degeneration of the alpha cells, yet has little or no influence upon the blood sugar (Volk, Lazarus and Goldner, 1953). Synthalin A brings about an inconstant hydropic vacuolation and degranulation of some alpha cells, but initiates a profound hypoglycemia.

We believed that some of the ambiguities may be reduced if the procedures originally used in the identification of HGF were carried out upon pancreatic tissue from animals previously injected with these cytotoxic compounds. Moreover, further evidence would be available to support

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or refute the previous claim by one of us that a 2-stage chemical nesidiectomy had been successfully accomplished (Fodden, 1953b).

EXPERIMENTAL METHODS

The rabbit, of 2 to 3 Kg. weight, was the experimental animal used.

Series 1. Cobaltous Chloride and Synthalin A on HGF of Normal Rabbits.

Six rabbits were killed by massive-air-embolus and their pancreas removed immediately, covered, and placed upon solid CO_2. Six rabbits were given intravenous injections of cobaltous chloride, 50 mg. per Kg. as a 5% solution in normal saline (Fodden, 1953a). After 12 hours, a time known to be adequate for maximum alpha cell injury (Fodden, 1953a; Volk, Lazarus and Goldner, 1953), they were killed and their pancreas removed. Six more animals received injections subcutaneously of an aqueous solution of synthalin A in 10 mg. per Kg. dosage (Davis, 1952; Fodden, 1953b). Hourly blood sugar determinations showed the progress of their hypoglycemia, and when convulsions began after approximately 6 to 10 hours, the rabbits were killed and their pancreas removed.

Series 2. *Cobaltous Chloride and Synthalin A on HGF of alloxan-diabetic rabbits.* The same procedures were repeated upon alloxan-diabetic rabbits exhibiting a consistently elevated blood sugar, but whose metabolism was stable without the use of insulin. None showed ketonuria or ketonemia. Six rabbits served as alloxan-diabetic controls, six were given Synthalin A., and six cobaltous chloride. All were followed during their subsequent course by blood glucose determinations at 45-minute intervals and frequent tests for ketonuria and ketonemia. The appearance of ketone bodies was noted in 5 animals of only the synthalin A group.

The method described by Sutherland and deDuve (1948) was used to prepare the pancreatic extracts for the hyperglycemic-glycogenolytic factor. The process of reextraction with the 2.5 volumes of acid alcohol was allowed 2 hours instead of 1, as was suggested by Audy and Kerly (1952). The final clear dialyzates from the non-diabetic series were divided into two equal portions. One portion was retained while the other was incubated anaerobically for 12 hours with cysteine to inactivate the insulin (Sutherland and deDuve, 1948; Sutherland, Cori, Haynes and Olsen, 1949). The liver slices were prepared from rabbits bled to death. The liver was immediately removed and placed in a chilled petri dish set in cracked ice. Liver slices approximately 0.6 mm. thick and 1 sq. cm. in cross section were cut with the fresh-tissue microtome described by Martin (1942). Selected slices were weighed on a Roller-Smith precision balance and dropped in 3 ml. of chilled chloride phosphate buffer in Warburg flasks. Where extract was added, the flasks contained 2.75 ml. of buffer and 0.25 ml. of the pancreatic extract representing 250 mg. of original pancreatic tissue. The flasks were shaken at 110 oscillations per minute in a water bath at 37° C. for 40 minutes. At the end of the incubation period 2 ml. of the medium was removed, placed in 25 ml. Erlenmeyer flasks, and deproteinized by the addition of 1 ml. each of 0.3 N $Ba(OH)_2$ and 5 per cent $ZnSO_4$. After filtration, 2 ml. of the filtrate was used for glucose estimation by the method of Somogyi (1945).

The hyperglycemic property of the extracts was examined by injecting 2 Kg. rabbits intravenously with quantities of 2 ml. and determining blood glucose values at 5-minute intervals for 45 to 60 minutes.

RESULTS

Figures 1, 2, and 3 show the hyperglycemic effects of the extracts from animals of Series 1. The hyperglycemic-glycogenolytic factor was present

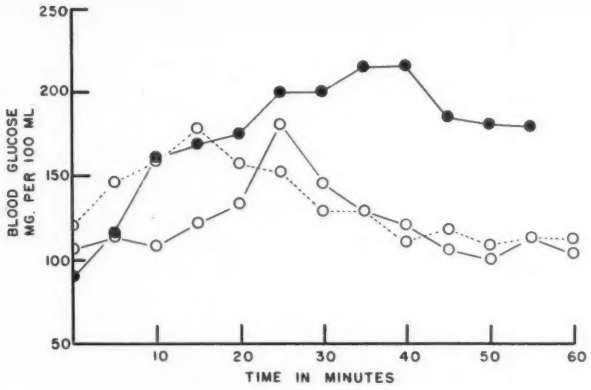

FIG. 1. The hyperglycemic effect of pancreatic extracts prepared from rabbits. 2 ml. of extract injected intravenously into normal rabbits.
○———○, Pancreatic extract of normal rabbits.
○ - - - ○, Cysteine-incubated pancreatic extract of normal rabbits.
●———●, Pancreatic extract of alloxan-diabetic rabbits.

in extracts from normal pancreas and also in those from the pancreas of animals previously treated with cobalt. Its presence was more readily apparent in the extracts which were incubated with cysteine; the hyperglycemic trend produced was then unopposed by the inactivated insulin (Fig. 2). When the animals had previously been given synthalin A, the hyperglycemic property of the resultant pancreatic extract was abolished (Fig. 3). Only the hypoglycemic effect of insulin remained, and when this was destroyed by cysteine inactivation, the blood glucose stayed unaltered. The results seen in Figure 4 occurred after the injection of a large dose of pancreatic extract from synthalin A-treated normoglycemic rabbits of another, earlier, experimental group. The extreme and unopposed insulin activity is evident from the levels to which the blood glucose fell. It was interesting to note that animals so treated could recover from the violent repeated convulsions at the blood glucose concentrations of 15 mg.%.

In the alloxan diabetic rabbits of Series 2, the pancreatic extract yielded by the control animals produced a hyperglycemic response of a sustained type. The features of its plateau-like graph, shown in Figure 1, were similar to those reported earlier by Sutherland and deDuve (1948). The alloxan-diabetic animals which had been given cobaltous chloride were the source of pancreatic extracts of remarkable hyperglycemic potency (Fig. 2). Those which had received synthalin A provided extracts which had no hyperglycemic activity (Fig. 3). Neither was an insulin reaction obtained by their use, nor of course was it anticipated.

The results of the investigations on the *glycogenolytic* principle are shown

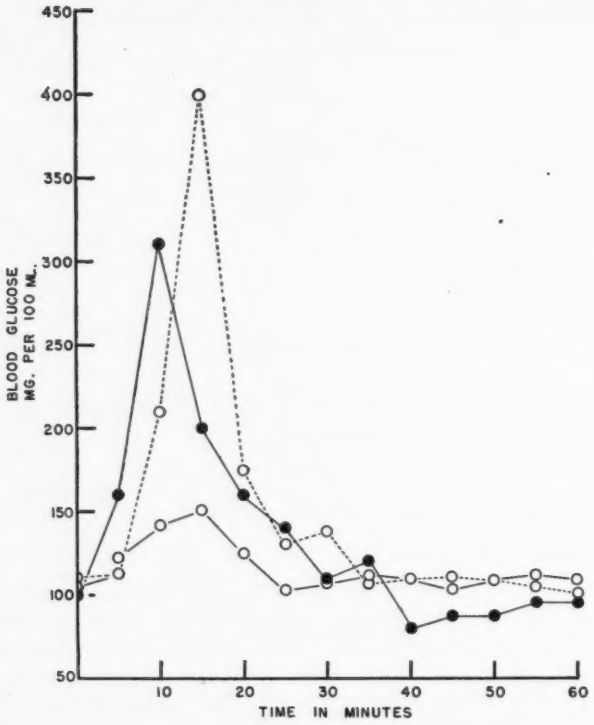

FIG. 2. The hyperglycemic effect of pancreatic extracts prepared from cobalt-treated rabbits. 2 ml. of extract injected intravenously into normal rabbits.

○———○, Pancreatic extract of cobalt-treated rabbits.

○ - - - ○, Cysteine-incubated pancreatic extract of cobalt-treated rabbits.

●———●, Pancreatic extract of cobalt-treated alloxan-diabetic rabbits.

in Figure 5. The untreated fraction of the extracts from normal pancreas initiated a glucose output from liver slices which amounted to 15% above the control slices. However, when the insulin had been inactivated, the glucose output rose to 100% above control slices. The same degree of stimulation of glucose output was exhibited with extracts from the control alloxan-diabetic animals, which gave, again, 100% increase in glucose output when compared to controls. It was noted that considerable variations could occur in the glycogenolytic activity of pancreatic extracts from alloxan-diabetic animals when maximum beta-cell destruction was uncertain. Thus, it appears that insulin must antagonize the liberation of glucose by the HGF of normal pancreas even in the simple incubating medium employed here.

After the maximum histologic changes in the alpha cells had ensued upon

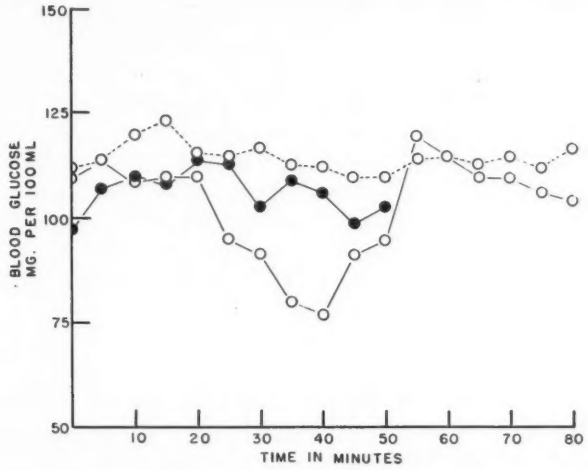

FIG. 3. The absence of the hyperglycemic effect of pancreatic extracts prepared from synthalin A-treated rabbits. 2 ml. of extract injected intravenously into normal rabbits.
○———○, Pancreatic extract of synthalin A-treated rabbits.
○ - - - ○, Cysteine-incubated pancreatic extracts of synthalin A-treated rabbits.
●———●, Pancreatic extracts of synthalin A-treated alloxan-diabetic rabbits.

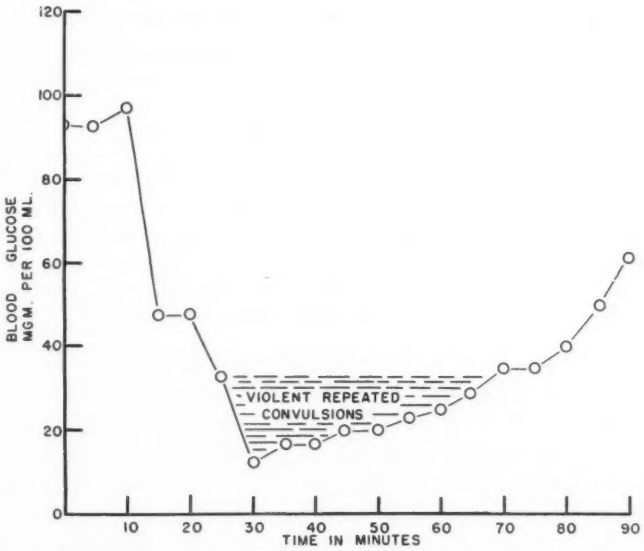

FIG. 4. The hypoglycemic effect of a pancreatic extract prepared from synthalin A-treated rabbits. 4 ml. of extract injected intravenously into a normal rabbit.

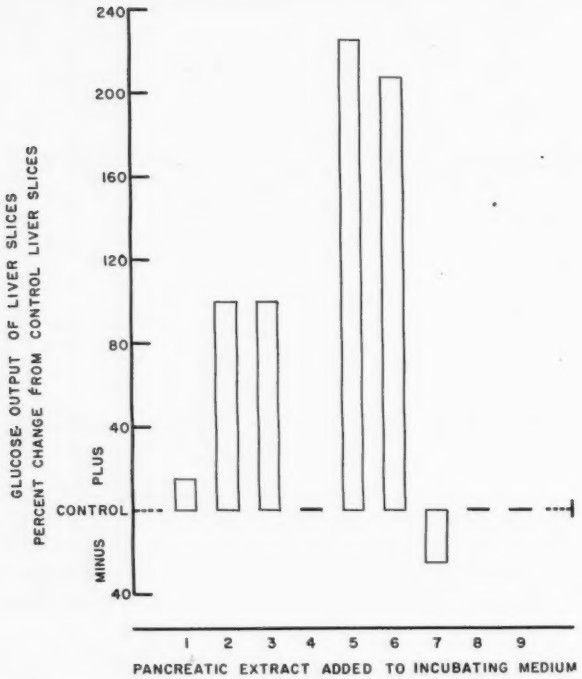

FIG. 5. The glycogenolytic activity of pancreatic extracts from treated rabbits, on liver slices.

1. Pancreatic extracts of normal rabbits.
2. Cysteine-incubated pancreatic extracts of normal rabbits.
3. Pancreatic extracts of alloxan-diabetic rabbits.
4. Pancreatic extracts of cobalt-treated rabbits.
5. Cysteine-incubated pancreatic extracts of cobalt-treated animals.
6. Pancreatic extracts of cobalt-treated alloxan-diabetic rabbits.
7. Pancreatic extracts of synthalin A-treated rabbits.
8. Cysteine-incubated pancreatic extracts of synthalin A-treated rabbits.
9. Pancreatic extracts of synthalin A-treated alloxan-diabetic rabbits.

cobaltous chloride injection, the glycogenolytic activity of these extracts showed no change in glucose output relative to control liver slices. However, after the insulin in this extract had been inactivated with cysteine there was a 225% increase in glucose output compared to the control liver slices, a figure representing roughly twice the amount of glucose liberated by unopposed HGF from normal pancreas.

The extracts prepared from the alloxan-cobalt rabbits produced the same phenomenon. The glucose release from liver slices was approximately twice

that of alloxan-diabetic animals. This indicates that a greater amount (approximately 2×) of the glycogenolytic principle can be extracted from cobalt-treated animals than from normal animals.

The extracts from the pancreas of synthalin A-treated animals produced a 25% decrease in freed glucose; essentially, a measure of the glycogenic activity of insulin. This was clearer from the experiment in which the insulin of this extract was inactivated by cysteine. The glucose output was then equal to that of control liver slices. Extracts from the alloxan-diabetes-synthalin A group gave a glycogenolytic activity only equal to the control slices. The inference must be that both the HGF and insulin had been destroyed.

DISCUSSION

Although HGF has not yet fulfilled all the criteria of a hormone (Vincent, 1922), we can accept as established its presence in pancreatic extracts of numerous animal species. Aware of its properties, we could possibly speculate that the normal levels of blood glucose may largely represent the influence of a balanced product of beta- and alpha-cell secretion. Interference with one will result in a relative over-abundance of the other. Synthalin A seems to inhibit completely the hyperglycemic-glycogenolytic function of the islet tissues, whilst leaving undisturbed the insulin activity of the beta-cell component. Indeed, the insulin potency after the use of this compound appears enhanced. Where alloxan and synthalin A have been combined, the resultant pancreatic extract has no different action from a phosphate-chloride buffer solution.

We must believe that cobaltous chloride does not cause the functional incapacity of the alpha cells that histologic appearances would suggest. Indeed, rather than any adverse interference with these structures, it produces the semblance of enhanced activity. This fact, taken together with the histology of the majority of the altered alpha cells being one of cellular swelling, edema, and vacuolation (Fodden 1953b; Van Campenhout and Cornelis, 1951) possibly indicates a cellular retention of active material. We make the suggestion that in some manner cobalt prevents the release of HGF, with its consequent intracellular accumulation; larger amounts would be subsequently extracted by the procedures we used, a phenomenon which we found to exist. This matter is now being investigated with radioactive cobalt.

We believe also that these experiments support the concept that the site of production of the hyperglycemic-glycogenolytic factor is the pancreatic islet-tissue alpha cells.

SUMMARY

Extraction procedures upon pancreas for the alpha-cell factor (HGF) were used to estimate the function, or secretion of this cell following the administration of cobaltous chloride and synthalin A.

These chemical compounds cause histologic alterations to the pancreatic insular alpha cells in the living rabbit.

Our conclusions are, that synthalin A abolishes the extractable hyperglycemic-glycogenolytic factor of the pancreas completely; cobaltous chloride increases the amount of extractable HGF from the pancreas.

Acknowledgments

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COMPARISON OF THE EFFECTS OF GONADECTOMY ON SPONTANEOUS ACTIVITY OF WILD AND DOMESTICATED NORWAY RATS[1]

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THE results of previous studies demonstrated that in the Norway rat, domestication has greatly changed the anatomy and physiology of the adrenal glands. In the domesticated form these glands are much smaller (Rogers and Richter, 1948); they contain less cholesterol and ascorbic acid (Nichols, 1950; Woods, in press); histochemically they show less lipid in all zones (Mosier, in press); and their removal has much less effect than in the wild form, since domesticated Norways survive adrenalectomy on only small amounts of added salt, whereas wild rats do not survive on salt in any amounts or form, and not consistently even on treatment with cortisone or DOCA (Richter, Rogers and Hall, 1950; Covian, 1949).

A study has now been made of the effects of domestication on the anatomy and physiology of the gonads.

Comparison of the effects of gonadectomy on sex behavior as not possible since wild rats are so suspicious that they will not mate when under observation.

For this reason our studies were largely limited to the effects of gonadectomy on spontaneous running activity which can readily be recorded even for the most suspicious wild rats. Wang (1923), Hoskins (1925) and others have shown that gonadectomy makes domesticated rats almost totally inactive; we were interested to know whether it has the same effect on wild rats.

METHODS

All-metal cages used for measuring spontaneous running activity were modified from those described in detail elsewhere (Richter and Wang, 1926). There are two parts: a revolving drum, 13 inches in diameter and 6 inches wide, and a small cage 3.5×5×11 inches, which serves as a living compartment. The cage hangs from two hooks

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on one side of a metal partition; the drum is mounted on a bicycle hub on the other side; a 3 inch circular hole in the partition joins the two. The living compartment contains a non-spillable food-cup and a graduated inverted water bottle with a 100 cc. capacity. A cyclometer registers each full revolution of the drum in either direction.

The drums were tested for standard ease of rotation every 2–3 weeks. Stock diet[2] and water were available *ad lib.* Food and water intake as well as activity, were recorded daily. The domesticated rats were weighed weekly, the wild rats bi-weekly.

Forty-one rats were used in these experiments; 26 wild rats (13 males and 13 females) and 15 domesticated rats (7 males and 8 females). Of the 26 wild rats, 12 were trapped in the yards and cellars of houses, and 14 were born in the laboratory to captured pregnant mothers. The trapped experimental rats were very young and probably had just been weaned. They were placed in the activity cages and on our stock diet within 3 days after capture. The rats born in the laboratory had access to the stock diet while they were still nursing, and were placed in the activity cages shortly after weaning. The 15 domesticated rats came from our colony that has been in existence for over 30 years. They too were put into the activity cages at an early age. Thus the wild and domesticated rats shared the same environment and diet throughout most of their post-natal lives.

For the first few days in the cages the rats remain quite inactive. After that their activity increases, often very rapidly, until it attains a fairly constant level. In these experiments the domesticated rats reached this level in 35–60 days after starting in the cages; wild rats after 20–50 days.

The gonads were not removed until activity had attained a constant level. At gonadectomy the domesticated rats weighed more than the wild rats, not only because they needed more time to reach the constant level in activity, but also because they grew at a more rapid rate in the first 4 to 8 weeks after birth.

Of the 13 wild males, 6 were castrated and 7 were controls; of the 13 wild females, 4 were spayed and 9 were controls. Of the 7 domesticated males 4 were castrated and 3 were controls; of the 8 domesticated females, 4 were spayed and 4 were controls.

The domesticated rats were sacrificed 50 days after gonadectomy, the wild rats after 100 days. At autopsy the organs were removed, weighed and preserved for histological and histochemical studies. Adrenals were frozen, sectioned and stained with Sudan IV.

The wild rats were captured in box traps (Richter and Emlen, 1945). They were brought to the laboratory in holding cages and then changed to the activity drums by means of small transfer traps. For removing the rats from the activity cages we used the "Emlen sock" (Emlen, 1944), a

[2] Graham flour 72.5%, casein 10.%, skim milk powder 10.0%, butter 5.0% calcium carbonate 1.5%, sodium chloride 1.0%.

device in which rats can safely be weighed, tube-fed or etherized. Under ether the rats can safely be tied down to the operating board and handled with the same methods used on domesticated rats.

RESULTS

Spontaneous Running Activity

Figure 1A shows a typical record of the running activity of a domesticated Norway female rat before and after gonadectomy. The ordinates give running activity in number of revolutions of the drum, body weight and food intake in grams; the abscissas age in days. This rat was placed in the activity cage when 43 days old; spayed at 105 days; and sacrificed at 155 days.

The record shown for the 32 days preceding gonadectomy is typical for young adult domesticated females. The running activity averaged about 16,000 revolutions per day and showed marked and regular fluctuations

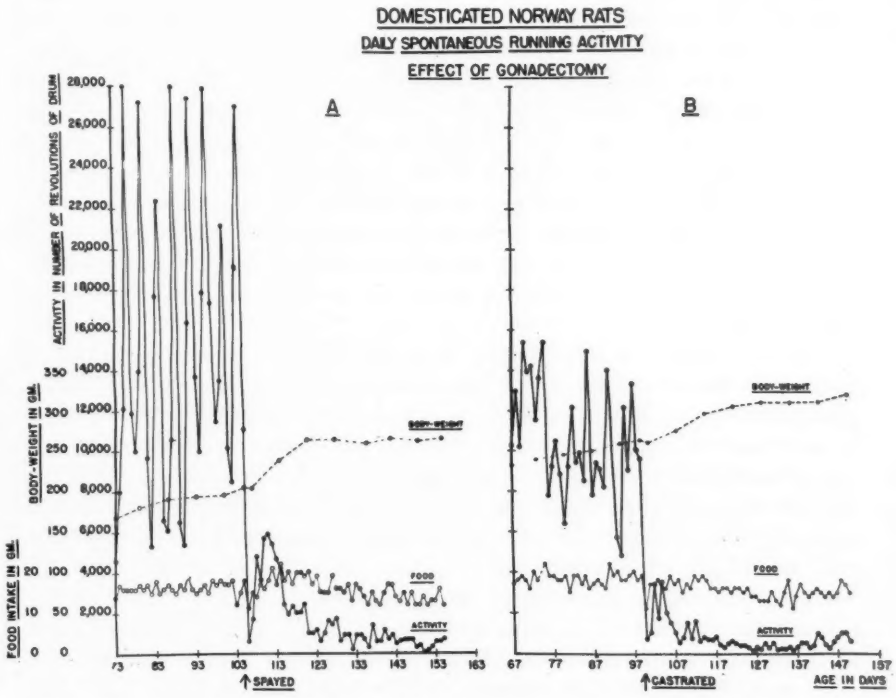

FIG. 1. Typical graphs showing the effects produced by gonadectomy in domesticated Norway rats: A) female; B) male.

every fourth day with maxima corresponding to days of ovulation (as determined by Wang (1923) in studies correlating vaginal smears and running activity). From observations of hundreds of normal domesticated rats, it can be assumed that without operation this rat would have remained just as active for many months; likewise that its food intake would have remained constant near 16 gm. per day and its body weight would have continued to increase at a slow rate.

Spaying resulted in a sharp decrease in activity to a very low level lasting two days, then in a temporary return to a slightly higher level lasting about 15 days, and finally in a less abrupt decrease to a state of almost complete inactivity. Food intake first increased to a definitely higher level for about 20 days, then decreased slightly below the normal preoperative level. A striking increase in body weight reflected the increased food intake of the first postoperative period, followed by a leveling off but no loss of weight with the subsequent reduction of food intake and activity.

Figure 1B shows a record from one of the domesticated males before and after gonadectomy. Males are generally less active than the females and do not show the 4–5 day cycles of activity. This rat was started in the activity cage at an age of 37 days and castrated at 99 days. Its chart show the record from 67 days of age. During the 32 preoperative days charted, the daily activity showed relatively small fluctuations as compared with the female and averaged about 10,000 revolutions per day. Food intake remained constant near 19 gm. per day; body weight increased at a slow rate. Castration was followed by a sharp decrease in activity; subsequently there was a brief and partial increase, but after a week activity decreased to a permanently low level near 300 revolutions per day. After castration food intake gradually decreased and body weight increased at a more rapid rate.

Figure 2 shows a typical record for a wild Norway rat before and after castration. This male was started in the activity cage at an age of 26 days, castrated at 56 days, and sacrificed at 157 days. On its first day in the cage, it ran about 1000 revolutions. During the next 30 days it became progressively more active, reaching an average level of 14,000 revolutions during the last 10 days before operation. On the basis of results of previous studies on wild rats, we knew this rat would have maintained much the same activity range for many months.

Castration failed to produce the sharp drop in activity seen in the domesticated rats. Activity remained at essentially the same level as before castration. A temporary reduction in the amplitude of the daily fluctuations constituted the only change. Weight increased at about the same rate for 40 days postoperatively, before leveling off well below the usual

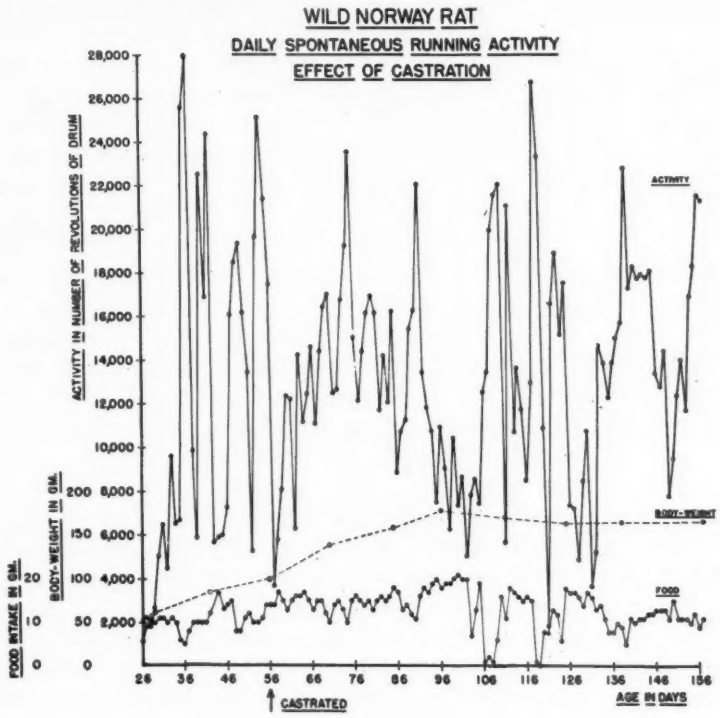

FIG. 2. Typical graph showing the effects produced by gonadectomy in a wild Norway rat.

weight of an adult wild Norway male. Food intake showed a slight increase during the first 50 days after operation, then a gradual decrease.

Figure 3 summarizes the effects of gonadectomy on the spontaneous activity of gonadectomized wild and domesticated rats. The ordinates give daily running activity in 10 day averages before and after gonadectomy; the abscissas give time in days. Figure 3A gives the average activity curves for 4 wild females and 4 domesticated females. In the last 10 days before spaying the two groups were almost equally active. After spaying the domesticated rats became quite inactive, averaging less than 1000 revolutions per day, whereas the wild rats became only slightly less active than before, averaging more than 8000 revolutions per day for the period; 40–50 days postoperative. The wild rats were still just as active at the end of 100 days. Within each group the results were consistent: all 4 domesticated rats showed a sharp decrease in activity; all 4 wild rats remained quite active.

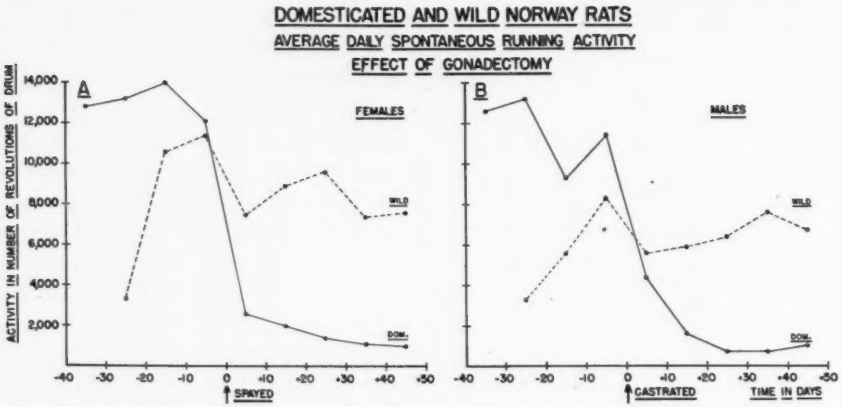

FIG. 3. Graphs showing the difference in effect of gonadectomy on spontaneous activity in domesticated and wild Norway rats.

Figure 3B summarizes the observations made on the effects of castration on 6 wild and 4 domesticated males. Here again, gonadectomy sharply reduced the activity of the domesticated rats, but produced little change in the activity of the wild rats. Fifty days later the daily activity for the wild rats averaged over 7000 revolutions whereas the activity of the domesticated rats averaged well under 1000. The wild males were still just as active at the end of 100 days. Within each group the results were consistent: all 4 domesticated rats showed a sharp decrease to a permanently low level; 5 out of 6 wild rats showed no change, 1 a slight decrease.

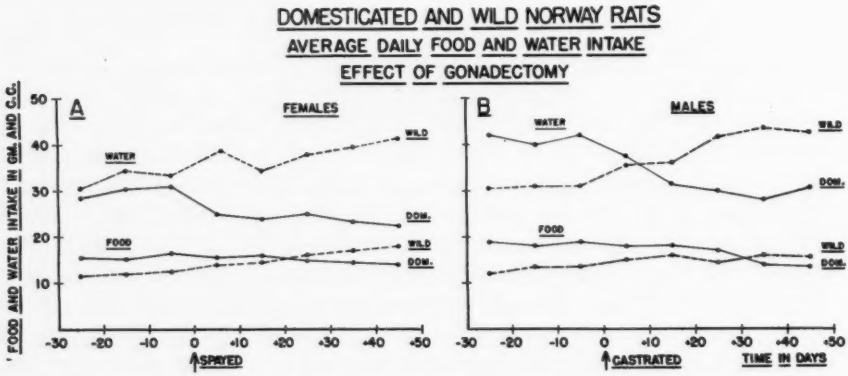

FIG. 4. Graphs showing differences in effect of gonadectomy on food and water intake in domesticated and wild Norway rats.

Food and Water Intake

Figure 4 summarizes the observations on the effects of gonadectomy on food and water intake of wild and domesticated rats, represented by interrupted and solid lines respectively. After gonadectomy the domesticated rats ate and drank progressively less, whereas the wild rats continued to increase their food and water intake at the preoperative rates.

Body Weight

Figure 5A shows the average body weight at 10 day intervals for 4 wild and 4 domesticated spayed females and for 9 wild and 4 domesticated control females. Data for wild rats are connected by broken lines; for domesticated rats by solid lines. Ordinates give body weight in grams, abscissas time in days (for 30 days before and 50 days after spaying). The curves for the domesticated rats show that for the 30 day preoperative

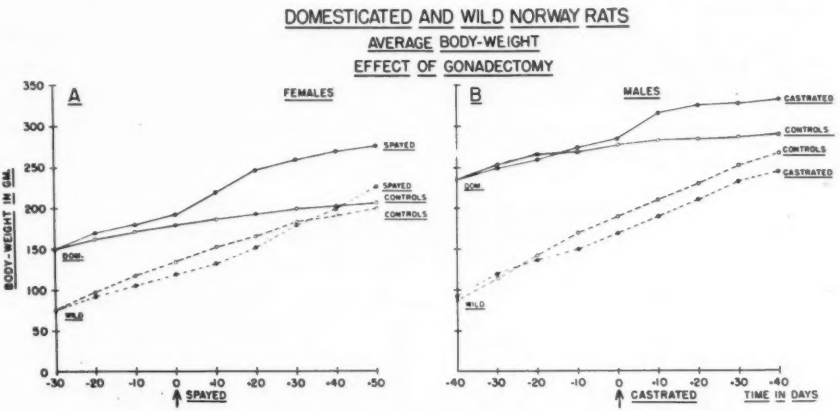

FIG. 5. Graphs showing differences in effect of gonadectomy on body weight in domesticated and wild Norway rats.

period the control and experimental groups had nearly the same average weights. The spayed rats gained weight at a much more rapid rate, so that after 50 days they weighed an average of 75 gm. more than the controls. In contrast, the control and spayed wild rats showed only minimal differences in weight.

Figure 5B shows that after castration, domesticated males similarly made a rapid gain in weight whereas the wild rats gained at the same rate as the unoperated controls.

Autopsy Weight

The bargraph in Figure 6 shows the average weights at autopsy for the seminal vesicles (with coagulating glands) and for the uteri in gm./kg. body weight; and for the adrenal and hypophyses in mg./kg. body weight. The solid and open bars represent the controls and gonadectomized rats respectively. The bars for the domesticated rats are indicated by a "D," those for wild rats by a "W."

Figure 6A shows that the seminal vesicles decreased in weight from 4.0 to 0.2 gm./kg. body weight for the domesticated rats; and from 5.3 to 0.05 gm./kg. body weight for the wild rats.

Figure 6B shows that the uteri of the domesticated rats decreased from 3.6 gm./kg. body weight to 0.7; whereas the uteri of the wild rats decreased from 2.0 gm./kg. body weight to 0.1. The greater atrophy of the uteri in wild rats, as well as of the seminal vesicles, may be accounted for in part by the additional 50 days that they were allowed to live after gonadectomy.

Figure 6C shows that gonadectomy had little effect on the small adrenals of the domesticated males, but was followed by a marked increase in the weight of the already large adrenals of wild males; in contrast, there was a definite decrease in the weight of the adrenals in the domesticated females, but only a doubtful increase in the case of the wild females. It will be

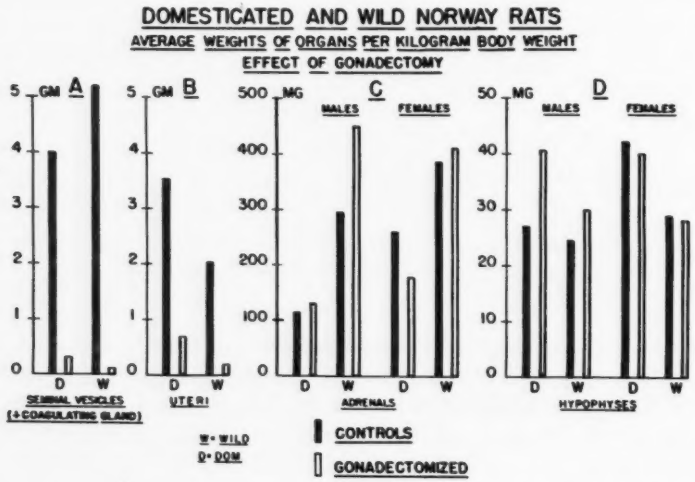

FIG. 6. Bargraph showing average weight of seminal vesicles, uteri, adrenals and hypophyses of normal and gonadectomized domesticated and wild Norway rats. Approximate age of rats, 150 days.

noted that after gonadectomy, the adrenals from the wild males actually out-weighed those from the wild females, whereas the opposite is invariably the case in normal rats.

Figure 6D shows that preoperatively the hypophyses were larger in domesticated than in wild rats. After gonadectomy the weights of the hypophyses increased in both wild and domesticated males, but to a much greater degree in the latter; they decreased negligibly in the females of both forms.

It will be noted that in both wild and domesticated rats, sex differences in size of the hypophyses and the adrenals were nearly lost.

Histological and histochemical studies so far have been made only of the adrenal glands. The adrenals of gonadectomized wild rats had large amounts of Sudan IV stained lipids, possibly even more than the controls. In marked contrast, the adrenals of the gonadectomized and control domesticated rats had very little lipid.

DISCUSSION

How can we explain the great decrease in activity after gonadectomy in the domesticated rat and the relative absence of any such change in the wild rat?

A possible explanation is that the wild rat may have accessory gonadal tissue or receive gonadal secretion from the adrenals or other sources. This explanation occurred to us because domesticated gonadectomized rats treated with sex hormones, or with ovarian or testicular implants, can be kept normally active (Wang, Richter and Guttmacher, 1925; Bugbee and Simond, 1926; Hemingsen and Kraray, 1937). Against this explanation is our finding that the secondary sex organs of the wild rat were just as much atrophied as those of the domesticated rats. There were no other signs of the presence of female or male hormones.

Another explanation is based on the assumption that steroids from the adrenals as well as from the gonads may contribute to the production of spontaneous running activity. The wild rats' adrenals would be expected to contribute higher amounts of steroids since they are larger and by various tests show greater secretory activity than those of the domesticated rats. The fact that gonadectomy has so little effect on the running activity would thus indicate that in this strain most of the steroids that produce running activity come from this gland. The domesticated rats' small adrenals show much less secretory activity and so would be expected to contribute less to the production of spontaneous running activity. The fact that gonadectomy has such a profound effect on spontaneous activity of domesticated rats would indicate that in this strain activity depends

largely on the steroids from the gonads. In agreement with this formulation is the observation that the sharp drop in activity that gonadectomy produces in domesticated rats can be either entirely or partially prevented by replacement therapy with adrenal hormones, cortisone or DOCA (Richter, 1951).

The results of these experiments showing that gonadectomy has so much greater effect in the domesticated than in the wild rats, may be interpreted to mean that during the process of domestication the gonads have come to play a relatively more important part in the rats' life. We should like now to present a few other observations from this laboratory and elsewhere that are in agreement with this interpretation:

1) Comparisons of organ weights have shown that the secondary sex organs, the uteri, ovaries and testes develop at an earlier age in domesticated than in wild rats (Richter, 1949).

2) In confirmation of observations made in other colonies of domesticated rats, we have found that during the 30 years of the existence of our colony, the vaginas of the females have tended to open at progressively earlier ages. In the early years of our colony, the vaginas did not open with any consistency until the rats were 70 days old and weighed approximately 140 gm. Now they open in some rats that are only 36 days of age and weigh 76 to 80 gm. They are open in all rats at 52 days of age and weighing from 104 to 126 gm.

Comparisons with wild rats can only be made on the basis of body weight, since the age of trapped rats is not known. Davis (1949) examined the vaginas of 1677 wild females, trapped in the city and weighing from 24–649 gm., and 1151 trapped on a farm and weighing 24–449 gm. The vaginas were not open in all rats until the weights reached 175–199 gm. In the 100 to 124 gm. range in which the vaginas were open in 100% of the domesticated rats, only 62% were open in 88 wild rats trapped in the city and 77% in 79 rats trapped on a farm.

3) The testes descend at an earlier age in domesticated rats. Donaldson (1924) reported the average age at which they descended in his colony was 40 days; in our colony the testes descend in some rats when they are less than 15 days of age and weigh only 20–26 gm.; we found that they had descended in all rats at an age of 24 days or when the rats weighed 54–62 gm.

As a result of studies made on 1432 wild males captured in the city, Davis (1949) reported that in wild rats the testes may descend when the rats weigh from 25–49 gm. (12%); that at weights of 50–74 gm. only 15% were descended; that they were descended in all, or in all except a few rats, only when the weights reached 250–274 gm. (96%). According to their calculations, the weights at which 50% of the rats on the farm had scrotal

testes was 136 gm. For 1002 city rats this weight was 119 gm.; for rats living indoors, 109 gm.

4) Mature domesticated rats from our colony as well as from other colonies show almost 100% fertility whereas according to Davis and Emlen (1948) many captured wild female fully grown adults were found to have never even given birth to young or to have given birth to only a small number of rats. This was established by noting the presence and number of "placental scars" on the uteri. In most instances only 25–40% were pregnant. The percentage of lactating females was even lower.

5) In our experience only a very small percentage of captured wild females show regular 4–5 day estrous cycles, as determined from activity records in revolving drums. In marked contrast a high percentage of domesticated rats show very regular 4–5 day estrous cycles.

6) Wild rats have a marked seasonal variation in their reproductive rate with high levels occurring in July, August and September, and low levels in November, December and January (Emlen, unpublished). Domesticated rats mate and reproduce quite freely throughout the year.

7) It has been reported that domesticated rats breed at progressively earlier ages during the process of domestication; and that they breed at an earlier age than the wild rats. Donaldson (1924) concluded: "the tendency of the female rats to breed at an earlier age has been noted in the Institute and the same was reported in other colonies."

Davis (1949) found only 4 out of 602 wild rats that were pregnant in the weight range below 200 gm.; and only 1 rat in this range that was lactating. Only 16% were pregnant in the 250–274 gm. group. The domesticated rats of our colony are mated for breeding when they are 150–200 gm. in weight and are about 90 days of age.

It will be interesting to know to what extent the differences found between the domesticated and wild Norway rats will be found also in other strains of rats and in other species of animals. We should like to know whether in other wild animals, gonadectomy likewise has little or no effect on spontaneous activity, and whether in other domesticated animals it has the same depressing effect on activity.

SUMMARY

1) Gonadectomy greatly reduced the spontaneous running activity of domesticated Norway rats, but had little effect on the activity of wild Norways.

2) After gonadectomy, the seminal vesicles and uteri atrophied in both the wild and domesticated rats.

3) The adrenals, which normally are heavier in wild rats, became even heavier after gonadectomy in wild males and less so in wild females; but de-

creased in domesticated females and showed a small increase in domesticated males. Sudan IV stained frozen sections showed that the adrenals of wild Norways, both control and gonadectomized, contained more lipid than those of domesticated rats.

4) In explanation of the differences of the effects of gonadectomy in the two strains, it was suggested that running activity of wild rats is maintained largely by adrenal secretions whereas the activity of domesticated rats is maintained largely by gonadal secretions.

5) The results indicate that during the process of domestication of the Norway rat, the gonads may have assumed the control of functions that are predominantly controlled by the adrenal in the wild state.

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CORRELATION OF OVARIAN CHOLESTEROL LEVELS WITH CHANGES IN β-GLUCURONIDASE ACTIVITY OF REPRODUCTIVE TRACT DURING THE ESTROUS CYCLE AND PREGNANCY[1,2]

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PROFOUND alterations in mass and composition of the uterus and associated structures during reproductive cycles in the rodent are well established. Relatively little attention has, however, been directed toward analysis of these processes at the metabolic level.

The association of changes in β-glucuronidase activity of certain tissues with both fluctuations in hormonal environment (Fishman, 1947) as well as active proliferation (Levvy, Kerr and Campbell, 1948; Kerr, Campbell and Levvy, 1949, 1950) has been pointed out. The events accompanying the estrous cycle and pregnancy would appear to provide the appropriate circumstances required to test this dual hypothesis of the physiological significance of the enzyme. Accordingly, a portion of the present study is concerned with the determination of β-glucuronidase activity of the uterus, preputial and mammary glands and liver in the rat in the course of the stages of the estrous cycle and at intervals during gestation.

In view of the accumulated evidence to support the hypothesis that mobilization of cholesterol from the corpora lutea and residual (interstitial) ovary of the rat parallels phases of heightened secretory activity of these structures (Dempsey and Bassett, 1943; Everett, 1945, 1947; Claesson and Hillarp, 1946; Levin and Jailer, 1948; Aldman, Hillarp and Odeblad, 1949a, b), the cholesterol content of the corpora lutea and residual ovary was determined during the various phases of reproductive activity in an attempt to obtain indirect evidence of their secretory activity.

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[2] A portion of these data was taken from a thesis submitted by Arthur L. Beyler to the Graduate Division, University of California, Los Angeles, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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METHODS AND MATERIALS

Female albino rats of the Sprague-Dawley strain and female CBA mice were employed in these studies. Rockland pellets and water, supplemented with fresh lettuce twice weekly, were fed *ad libitum*. Whenever possible, litter mates were distributed uniformly among the various experimental groups.

Timing of the estrous cycle and pregnancy. Vaginal smears of young adult virgin rats were taken each morning over a period of two weeks. Only those rats exhibiting a regular $4\frac{1}{2}$ to 5 day cycle were used. Animals which showed typical diestrous, proestrous or estrous smears following the preliminary observational period were sacrificed within one hour after the vaginal smear was taken. Rats which were to be used for studies during the various stages of pregnancy were placed in mating cages so that 2 or 3 females were served by one male. Vaginal smears were taken each day and the first day of pregnancy was reckoned as the day when sperm were identified in the smears. Pregnant rats came to experiment on the 5th, 10th, 15th, or 20th gestational day.

Vital staining of the corpora lutea. Distinction of the corpora lutea formed subsequent to the most recent ovulation from those persisting from the second or even third ovulation removed is impossible for all practical purposes without some means of differentiation. Accordingly, in those experiments involving cyclic rats, one ml. of a 1% solution of the vital dye, Trypan Blue, in physiological saline was injected intraperitoneally on the 2nd diestrous day (cf., Long and Evans, 1922). The corpora lutea existing at this time stained a deep to a light blue depending upon their age (i.e., the more recently formed corpora lutea take up less stain). The rats were allowed to continue through the cycle and were sacrificed subsequent to the next diestrous period. The new corpora lutea which were formed after the ovulation following the dye injection were unstained and could be readily distinguished from the older and stained ones. This technique was resorted to only in the experiments involving cyclic rats. No difficulty was encountered in isolating the corpora lutea of pregnancy due to their much greater size in comparison to the other older and regressing corpora lutea present in the ovary during the earlier stages of pregnancy. The presence of the dye in the tissues, although found not to interfere with the cholesterol analyses of the ovary, was observed to add sufficient color to tissue homogenates to complicate the determination of β-glucuronidase activity. Therefore, the study of the enzyme activity of the reproductive tract during the estrous cycle was made on rats uninjected with dye.

Cholesterol determination. The procedure used for the determination of the free and combined cholesterol of the corpora lutea and the residual ovary was essentially that of Schoenheimer and Sperry (1934) with slight modifications as suggested by Sturges and Knudson (1938) for application to tissue analysis. It was necessary, particularly in the case of the corpora lutea during the estrous cycle, to adjust the volume of the reagents to a semi-micro scale in order to increase the sensitivity of the colorimetry. In making these adjustments care was taken to insure that the proportionate amounts of reagents, as outlined in the prescribed procedures, were maintained.

β-Glucuronidase determination. The assay of tissue β-glucuronidase activity was performed according to the method of Talalay, Fishman and Huggins (1946). This method involves the colorimetric determination of phenolphthalein liberated by the action of the enzyme on the biosynthetically prepared substrate, phenolphthalein-mono-β-glucuronide[4] (Fishman, Springer and Brunetti, 1948). One unit of activity is defined

[4] The substrate, phenolphthalein-mono-β-glucuronide as the cinchonidine salt, was purchased from the Sigma Chemical Co., 4648 Easton Avenue, St. Louis 13, Missouri.

as the amount of enzyme which will liberate one μg. of phenolphthalein from the substrate in one hour at a pH of 4.5 and at a temperature of 38° C. The enzyme activity in this report is expressed as the number of units per mg. of wet tissue.

RESULTS

Ovarian cholesterol during the estrous cycle and pregnancy. The free and esterified cholesterol values for the corpora lutea and residual ovary[5] during the estrous cycle and pregnancy are shown in Table 1. It was observed that the free cholesterol concentration for both components of the ovary was consistently uniform throughout all phases of reproductive activity investigated. This uniformity existed regardless of the rather abrupt changes which occurred in the weight of the residual ovary as observed between the estrous stage of the cycle and the 5th day of pregnancy, and also those alterations in growth of the corpora lutea between the 10th and

TABLE 1. FREE AND ESTERIFIED CHOLESTEROL CONTENT OF THE RESIDUAL OVARY AND CORPORA LUTEA DURING THE ESTROUS CYCLE AND PREGNANCY

Phase of reproductive activity	No. of rats	Residual ovary			Corpora lutea		
		Weight, mg.	Cholesterol (%)		Av. wt. each, mg.	Cholesterol (%)	
			Each	Esterified		Free	Esterified
Estrous Cycle:							
Diestrus	6	57.5	0.314±0.036[1]	0.749±0.057	1.02	0.241±0.027	0.776±0.065
Proestrus	5	58.7	0.311±0.015	0.726±0.055	1.21	0.304±0.065	0.587±0.015
Estrus	5	58.7	0.258±0.045	0.414±0.025	1.20	0.271±0.040	0.270±0.035
Pregnancy:							
5th day	6	52.1	0.305±0.037	1.11 ±0.11	1.64	0.192±0.027	0.262±0.040
10th day	8	44.1	0.354±0.053	1.13 ±0.079	1.78	0.289±0.072	0.272±0.065
15th day	5	44.2	0.200±0.025	1.29 ±0.16	4.42	0.271±0.030	0.342±0.034
20th day	5	52.3	0.296±0.050	0.881±0.13	4.40	0.277±0.045	0.314±0.035

[1] Results expressed as means and their standard errors.

15th day of pregnancy. On the other hand, in regard to the esterified cholesterol it was observed that a statistically significant diminution ($P < 0.01$) occurred between proestrus and estrus in both the residual ovary and the corpora lutea. During pregnancy the cholesterol esters of the residual ovary exhibited a sharp increase in concentration to the 15th gestational day after which time the concentration tended to return to that found on day diestrous. In contrast, the esterified cholesterol content of the corpora lutea during all stages of pregnancy examined remained at a depressed level. If we accept these changes in the esterified cholesterol concentration as indicative of the degree of secretory activity of the ovarian components (cf., Everett, 1945, 1947; Dempsey and Bassett, 1943; Claesson and Hillarp, 1946; Levin and Jailer, 1948; Aldman, Hillarp and Odeblad, 1949a, b), then it would appear that the residual ovary and cor-

[5] The term residual ovary as used in this report refers to that portion of the ovary which remained after the most recently formed set of corpora lutea had been removed.

pora lutea between proestrus and estrus of the cycle and the corpora lutea throughout the extent of pregnancy were more active than at any other observed time in secreting their steroid hormones.

β-Glucuronidase activity of the reproductive tract and other tissues during the estrous cycle and pregnancy. The β-glucuronidase activity of the uterus, preputial glands and liver during the estrous cycle and for these tissues plus the mammary glands during pregnancy is shown in Table 2. In the case of the liver and mammary glands only representative samples of the tissues were analyzed, and no record was kept of their total weight. At no time during the estrous cycle was the β-glucuronidase activity of the tissues found to fluctuate to any significant degree. The possibility that the increased water content of the uterus reported by Astwood (1938) to occur during late proestrus and early estrus might mask possible enzyme changes was ruled out by experiments in which one half of the uterus was used for enzyme activity determination while the remaining half was employed in determining the uterine water content. Under the conditions of these experiments the percentage of uterine water content was 81.1, 81.3 and 80.0 for diestrus, proestrus and estrus, respectively. From this it can be concluded that the uterine β-glucuronidase activity, whether expressed on the basis of wet or dry weight of the tissue, was found not to fluctuate during the various stages of the estrous cycle chosen for study. This confirms the observations of Leonard and Knobil (1950) who reported that the β-glucuronidase levels of the uteri of rats sacrificed at estrus and 60 hours postestrus showed no significant differences.

As seen in Table 2, however, marked fluctuations in uterine β-glucuronidase levels accompanied the stages of gestation. The activity of the uterine enzyme decreased abruptly with the onset of pregnancy. A statistically

TABLE 2.—β-GLUCURONIDASE ACTIVITY OF VARIOUS TISSUES DURING ESTROUS CYCLE AND PREGNANCY

Phase of reproductive activity	Uterus		Preputial glands		Liver	Mammary glands
	Wet weight, mg. 100 gm. BW	Enzyme activity	Wet weight, mg. 100 gm. BW	Enzyme activity	Enzyme activity	Enzyme activity
Estrous Cycle:						
Diestrus	131 ± 7.0* (7)	8.68 ± 0.56 (6)	23.1 ± 1.9 (6)	1707 ± 134 (6)	21.7 ± 1.8 (4)	—
Proestrus	186 ± 15 (4)	7.84 ± 0.45 (4)	22.2 ± 1.3 (4)	2214 ± 264 (4)	21.3 ± 1.8 (4)	—
Estrus	174 ± 22 (6)	7.82 ± 0.59 (5)	27.3 ± 3.0 (5)	2057 ± 156 (4)	23.4 ± 2.1 (5)	—
Pregnancy:						
5th day	211 ± 10 (6)	3.87 ± 0.39 (6)	33.4 ± 4.1 (6)	1555 ± 88 (6)	14.6 ± 4.1 (6)	2.69 ± 0.32 (6)
10th day	293 ± 11 (8)	2.11 ± 0.17 (8)	34.5 ± 2.9 (8)	986 ± 78 (8)	18.1 ± 1.6 (8)	2.17 ± 0.27 (8)
15th day	1027 ± 44 (5)	7.91 ± 0.59 (5)	31.2 ± 2.6 (5)	482 ± 65 (5)	17.7 ± 1.6 (5)	2.35 ± 0.21 (5)
20th day	1405 ± 92 (5)	6.32 ± 0.80 (5)	32.0 ± 2.0 (5)	1027 ± 110 (5)	22.3 ± 1.7 (5)	5.52 ± 0.28 (5)

* Results expressed as means and their standard errors.

significant difference ($P < 0.01$) occurred by the 5th gestational day and the activity continued to decline, reaching a minimum at the 10th day of pregnancy. From the 10th to the 15th day a pronounced upsurge in the enzyme activity of the uterus took place. This sharp rise paralleled the most rapid growth phase of the uterus as well as of the placentae, corpora lutea (cf., Table 1) and embryos. The uterine β-glucuronidase activity at the 20th day of pregnancy appeared to be on the decline; however, the value was not significantly different from that occurring at the 15th day. A remarkable correspondence of uterine β-glucuronidase activity and respiratory metabolism of this structure as a function of reproductive cycles has been observed (Roberts and Szego, unpublished data). The possible significance of this temporal correlation is not immediately apparent, and appears to require analysis of possible variations in other enzymes under similar conditions.

The preputial glands of the rat are characterized by inordinately high β-glucuronidase activity; nearly 100-fold greater than that reported for any, including neoplastic, tissue (Beyler and Szego, 1951). The significance of this observation in connection with the possible functions of these glands is discussed below. As in the case of the uterus, the β-glucuronidase activity of the preputial glands was also observed to be extremely responsive to the hormonal fluctuations accompanying pregnancy (Table 2). The pattern of the responsiveness agreed quite well with that of the uterus except for a slight lag. These glands did not exhibit a growth spurt between the 10th and the 15th day of pregnancy as did the uterus, placentae, corpora lutea and embryos, although the mean weight of these structures was found to be uniformly greater during the gestational period than found during the estrous cycle. The responsiveness of the preputial glands to castration and to exogenous steroids is discussed elsewhere (Beyler and Szego, 1954).

The mammary glands were included in the present study since it is well established that these glands respond dramatically to hormonal influences during pregnancy. This response is most pronounced in terms of growth and for this reason a study of the β-glucuronidase activity of these structures during pregnancy might be expected to shed some light on the role played by this enzyme in relation to growth processes. The β-glucuronidase activity of the mammary glands was observed to remain uniform throughout pregnancy except for the period immediately before parturition at which time a significant ($P < 0.01$) elevation occurred. That this increase did not parallel the most rapid growth phase of the mammary glands is indicated by the experiments of Roberts (1921) and of Weatherford (1929) who demonstrated that the mammary glands of the rat attain their maximum size during the first half of pregnancy.

The values for liver β-glucuronidase activity during pregnancy varied to a much greater extent than during the estrous cycle. There appeared to be a tendency for the liver enzymatic activity to become lowered by the 5th gestational day, although no degree of significance could be assigned to the differences. The apparent independence of the liver enzyme form hormonal influence under these conditions should not be construed to indicate its freedom from endocrine regulation. Marked effects have been observed in these laboratories through variation of pituitary and adrenal cortical hormone levels (cf., Beyler and Szego, cited in Szego and Roberts, 1953).

The influence of estradiol and progesterone on uterine β-glucuronidase activity. The finding that the uterine β-glucuronidase activity was greatly depressed during the first half of pregnancy (a period associated with increased progesterone synthesis, as suggested in the present investigation by the decreased levels of esterified cholesterol of the luteal tissue) prompted studies in which it was hoped that similar alterations in uterine enzyme activity could be reproduced in the castrate rodent by appropriate replacement of both estrogen and progesterone.

For this study ovariectomized CBA mice were brought to experiment on the 17th post-castration day. The animals were divided into three groups: control, estrogen injected and estrogen-progesterone injected. The estrogen injected mice received a total of 2.4 μg. of estradiol-17β[6] in 8% ethanol divided into 6 equal doses and injected subcutaneously over a 3 day period. The estrogen-progesterone treated mice received the same regimen of estradiol-17β plus a total of 0.6 mg. of progesterone in cottonseed oil divided into 3 equal doses and administered over a 3 day period. The control mice were injected with an equivalent volume of the solvents used. The animals were sacrificed by severing the cervical vertebrae 48 hours following the last injection and the uteri were assayed for their β-glucuronidase activity. The results of this experiment are depicted in Figure 1.

The administration of 2.4 μg. of estradiol-17β significantly increased ($P < 0.01$) the uterine β-glucuronidase activity of the ovariectomized mouse. A similar increase in activity of the uterine enzyme was noted in the castrate rat injected over a period of 3 days and observed 2 days later. This confirms the findings of Fishman and Fishman (1944), Fishman (1947), Kerr, Campbell and Levvy (1949, 1950) and Harris and Cohen (1951) for the mouse, and of Leonard and Knobil (1950) for the rat. The injection of progesterone together with the estradiol was observed to block completely the estrogen-stimulating effect on the uterine enzyme in the

[6] The crystalline steroids used in these studies were generously supplied by Dr. E. Schwenk of the Schering Corporation and by Dr. Robert P. Jacobson.

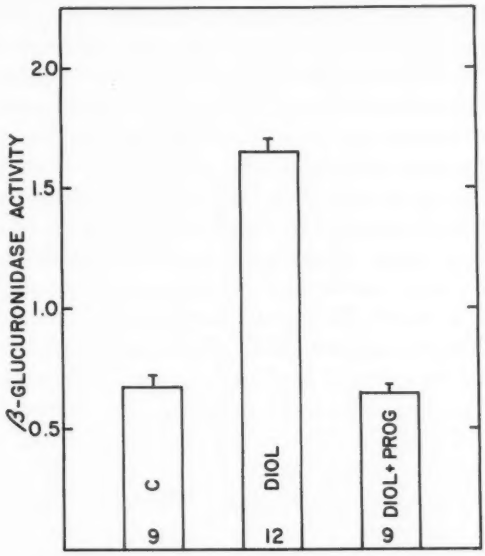

FIG. 1. *The influence of estrogen and progesterone treatment on uterine β-glucuronidase activity in the castrate mouse.* Where indicated, 2.4 μg. of estradiol-17β (DIOL) in 8% ethanol were given in 6 divided subcutaneous doses distributed over 3 days. In addition, a total of 600 μg. of progesterone (PROG) in corn oil was administered to one group in 3 subcutaneous injections over the same time period. The control group (C) received vehicles only. Autopsy was performed 48 hours after the last injection of estrogen. β-Glucuronidase activity is expressed as units of activity per mg. wet tissue (see text). Deviations shown are standard errors. The numbers of animals contributing to the analyses are represented by the figures at the base of the bars.

mouse. At the same time progesterone was found to have very little, if any, interfering influence on the estrogen-induced uterine growth. The mean uterine wet weight per 100 gm. body weight with their standard errors for the three experimental groups was 87.0 ± 3.1 for the control group, 194.4 ± 7.4 for the estrogen injected animals, and 161.5 ± 13.4 for the estrogen-progesterone treated mice.

DISCUSSION

The failure of the β-glucuronidase activity of the uterus and preputial glands to exhibit changes during the estrous cycle in association with altered estrogen synthesis by the ovary is difficult to reconcile with the hypothesis that this enzyme is intimately involved in the physiological mediation of estrogen activity as suggested by Fishman (1947). That the uterus itself is able to respond to the increased levels of circulating estrogen

during proestrus and estrus is readily seen upon gross inspection of this structure at this stage of the cycle. The increase in blood supply (Markee, 1932) and the alterations in osmotic behavior (Astwood, 1938) which occur during proestrus or early estrus are considered to be some of the earliest signs of estrogen stimulation. A possible explanation for the failure of the uterine β-glucuronidase values to reveal evidence of increased estrogen activity during the latter part of the estrous cycle (aside from the question of sensitivity of the enzyme) might be gleaned from an examination of the ovarian cholesterol levels. The esterified cholesterol of both the residual ovary and corpora lutea was found to diminish between the proestrous and estrous stages of the cycle. This would suggest that both estrogen and progesterone were being produced at an augmented rate at this period and that the effects of the estrogen in regard to uterine β-glucuronidase might be effectively counteracted by the "antagonistic" influence of progesterone. That progesterone is able to modify the estrogen stimulating effect on the uterine β-glucuronidase of the mouse has been cogently demonstrated in these and earlier studies (cf., Kerr, Campbell and Levvy, 1950; Harris and Cohen, 1951).

The abrupt decrease in the activity of the uterine respiration as well as β-glucuronidase observed to occur during the first 10 days of pregnancy is open to alternative interpretations: alteration in the ratio of the concentration of circulating estrogen and progesterone, or release of increased quantities of corticoids (Venning, 1946). The suggestion that the decline in the uterine enzymatic activity during the first part of pregnancy represents a response to an increased progesterone production with a concomitant tapering off of the estrogen levels is offered in view of the fact that the luteal esterified cholesterol values were observed to be low whereas those of the residual ovary were found to reach significantly higher figures during this phase of the gestational period. The return of the uterine β-glucuronidase activity on the 15th day of pregnancy to levels found to occur during the estrous cycle is more difficult to correlate with ovarian cholesterol changes, however. The cholesterol values for the ovarian components would indicate that progesterone production by the corpora lutea of pregnancy had not declined, nor the estrogen synthesis by the remainder of the gland increased, at this time. If it is assumed that the rise in uterine enzyme is a response to increased estrogen levels circulating in the blood, and if the cholesterol values can be used as a rough index to the degree of steroid hormone production by the ovary, then it must be assumed *a priori* that some other tissue present during pregnancy (possibly placenta) had reinforced the estrogen secretory function of the ovary with the net effect of increasing the E:P ratio.

The preputial glands of the rat were found to have the highest β-glucu-

ronidase activity of any tissue yet reported. On a weight for weight basis these glands possessed from 50 to 100 times the enzyme activity of the liver or the spleen. On a total organ basis the preputials contained from 50 to 80% of the number of units determined for the entire liver. The estimation by Mills (1946) that the liver and spleen contained approximately 60 and 25%, respectively, of the total β-glucuronidase activity of the rat requires revision if the extremely high preputial gland enzymatic level observed in this study receives confirmation. The rather meager information available concerning the physiology of these structures, together with the inability of many experiments (cf., Burrows, 1949) to establish an endocrine function for these glands, makes it difficult to assign any specific endocrinological significance to the findings reported here in regard to the fluctuations of preputial β-glucuronidase activity accompanying various reproductive phases in the rat. The fact that the preputial and uterine enzymatic activity responded in a similar manner to the hormonal changes accompanying pregnancy, however, would suggest that similar connotations may be placed on the function of β-glucuronidase in both tissues. The association of an increase in β-glucuronidase activity of several tissues with active proliferation of these structures (Levvy, Kerr and Campbell, 1948; Kerr, Campbell and Levvy, 1949, 1950) does not appear to exclude the alternative mechanism of "metabolic conjugation" cited for its action by Fishman (1947), although it is acknowledged that *in vitro* the enzyme appears to have primarily a hydrolytic function.

Evidence to suggest the possibility that the preputial glands might be involved in the metabolism of the steroids is offered by Montagna and Noback (1946) who reported that the secretion of these glands contained an abundance of steroids as revealed by histochemical techniques. The experiments of these authors suggest that the acinar cells of these glands synthesize the steroidal elements of the sebum. This suggestion, together with the extremely high β-glucuronidase activity of these structures, poses many provocative questions. Particularly germane questions which it is hoped future experiments will answer are these: Does an appreciable quantity of the steroid present in the preputial glands exist as glucuronide? If so, does the quantity of steroidal glucuronide fluctuate in accordance with altered levels of β-glucuronidase activity within these glands?

The hypothesis presented by Kerr and co-workers cannot be accepted as the sole explanation for the changes which occurred in the β-glucuronidase activity of the various tissues investigated in the study reported here. Refutation to the "growth theory" is offered by the following data: 1) The uterine β-glucuronidase activity declined during the first half of pregnancy while the uterus increased in size and weight. 2) A significant increase in the activity of the enzyme of the mammary glands took place

immediately prior to parturition, sometime after the maximum size of these structures had been reached. 3) The fluctuations in the preputial β-glucuronidase activity in no way paralleled the changes in the weight of these glands. 4) Although progesterone was demonstrated to prevent completely the rise in uterine β-glucuronidase activity of ovariectomized mice in response to administered estrogen, it did not prevent the estrogen-induced uterine growth.

One is inclined to believe on the basis of evidence available that the changes in the β-glucuronidase activity of the uterus, preputial and mammary glands in response to the fluctuations in the amount of circulating hormones during pregnancy or to the injected hormones, alone or in combination with progesterone and other steroids (Beyler and Szego, 1954), are a reflection of physiological action of the steroids, and that the activity of the enzyme need not necessarily be the limiting factor in steroid actions and interactions as Fishman (1947) has suggested. The striking correlation of the β-glucuronidase values with respiratory metabolism of the uterus (Roberts and Szego, unpublished data) would appear to support this view. The nature and significance of these phenomena in terms of the physiological action of the hormone as well as enzyme remain to be elucidated.

SUMMARY

The β-glucuronidase activity of the reproductive tract and associated structures of the female rat was studied together with the cholesterol content of the corpora lutea and residual (interstitial) ovary during the various stages of the reproductive cycle and for the 5th, 10th, 15th and 20th day of pregnancy. Decreased uterine and preputial gland β-glucuronidase activity during the first half of pregnancy occurred in conjunction with an apparent mobilization of esterified cholesterol from the corpora lutea of pregnancy and an accumulation of cholesterol esters in the residual ovary. This decline in the β-glucuronidase activity of the uterus and preputial glands was interpreted as representing a response on the part of the enzyme to the altered levels of circulating estrogens, progesterone and possibly adrenal steroids associated with the first half of pregnancy in the rat.

That the increased production of progesterone during pregnancy might account for the diminution in the uterine β-glucuronidase activity found to occur during the first 10 days of the gestational period was demonstrated in ovariectomized mice in which 0.6 mg. of progesterone was observed to block completely the elevating effect of 2.4 μg. of estradiol-17β on uterine β-glucuronidase activity.

The failure of changes in the β-glucuronidase activity of the uterus, preputial glands and mammary glands to correspond to phases of increased

growth of these structures was cited as evidence out of harmony with the "growth theory" action of this enzyme.

The significance of the extremely high levels of β-glucuronidase activity of the preputial glands was discussed in relation to the possible function of these modified sebaceous glands.

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STEROID HORMONE INTERACTIONS AS EVIDENCED BY MODIFICATION OF β-GLUCURONIDASE ACTIVITY OF THE PREPUTIAL GLANDS OF THE RAT[1,2]

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RECENTLY one of us (Szego, 1952) reported that 17-hydroxy-corticosterone (Compound F) and 11-dehydro, 17-hydroxy-corticosterone (Compound E) exhibited a marked inhibitory influence on the uterine water imbibition response (cf., Astwood, 1938) to simultaneously administered estrogen in the ovariectomized rat. The following additional steroids tested were negative in this regard: desoxycorticosterone acetate, testosterone, progesterone, and pregnenolone. Thus, considerable specificity in structural requirements was apparently involved in this evidence of steroid interaction.

The finding in this laboratory (Beyler and Szego, 1951) that the β-glucuronidase activity of the preputial glands of the rat was many times greater than that reported for any other, including neoplastic, tissue and that this enzyme activity fluctuated in apparent response to altered levels of circulating hormones accompanying pregnancy (Beyler and Szego, 1954) led us to investigate the β-glucuronidase activity of these modified sebaceous glands in relation to exogenous steroid treatment. The observations to be reported below reveal that this enzymatic activity in preputial glands is strikingly sensitive to hormonal influence. A single dose of estradiol-17β was found to double β-glucuronidase activity within 4 hours. It was further noted that those steroids which exerted antagonistic influence on the uterine water response to estrogen were effective as well in blocking estrogen stimulation of this enzymatic function in the preputial gland.

METHODS AND MATERIALS

The animals, materials and procedures used in this study were similar to those described by Szego (1952). Many of the experimental animals were identical to those reported in

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[1] Aided by a grant from the Cancer Research Coordinating Committee, University of California.

[2] A portion of these data was taken from a thesis submitted by Arthur L. Beyler to the Graduate Division, University of California, Los Angeles, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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the earlier study. Young adult virgin rats of the Sprague-Dawley strain were used 14 to 17 days after ovariectomy. All test materials were administered intravenously via the saphenous vein in the amount of 0.5 ml. per 100 gm. of body weight while the animal was under light ether anesthesia. Estradiol-17β in solution as the sodium salt in physiological saline was prepared according to the method outlined by Roberts and Szego (1947). The other steroids[4] employed were administered intravenously as fine crystalline suspensions. Wherever the steroids were given in combination, estradiol-17β was injected first followed immediately by the second.

The animals were sacrificed 4 hours following the injection. The preputial glands were excised, cleaned of adherent connective tissue and fat and weighed on a microtorsion balance. The β-glucuronidase activity was determined according to the method of Talalay, Fishman and Huggins (1946).

RESULTS AND DISCUSSION

The results of experiment are shown in Figure 1. It may be seen that 4 hours after a single intravenous dose of 0.5 μg. estradiol-17β per 100 gm. body weight, the β-glucuronidase activity of the preputial glands was virtually double the level in untreated castrate controls ($P < 0.01$), and similar to that seen in intact, cyclic females (cf., Beyler and Szego, 1954). This extremely rapid shift in enzymatic activity is taken toindicate changes in metabolic environment, possibly by virtue of estrogen-induced alterations in cellular permeability of "target" organs (cf., Szego and Roberts, 1953), rather than to reflect new enzyme synthesis. Compound E-acetate and Compound F-acetate completely blocked this estrogen effect ($P < 0.01$). These were the very same steroids observed to have a profound effect in preventing the increase in the uterine water content of similar animals as a consequence of estrogen administration (Szego, 1952). It will be noted further from Figure 1 that DCA, testosterone, progesterone and pregnenolone had no significant effect in modifying the estrogen influence on the preputial enzyme. These compounds were also inactive in preventing the estrogen-induced water increase (Szego, 1952) and other metabolic manifestations of estrogen activity upon the uterus (cf., Roberts and Szego, 1953). The present investigation demonstrates that strikingly similar structural requirements exist for adrenal steroid inhibitory influence on preputial β-glucuronidase response as well. As in the case of inhibition of certain uterine metabolic responses to estradiol, it is apparent that the 17-hydroxy group, together with the 11-oxygen function, on the steroid nucleus are essential before any measurable degree of "antagonism" is exerted

[4] The steroids used in these studies were generously supplied by Dr. Abraham White of Chemical Specialties, Inc., Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Dr. Molitor, Dr. Robinson, and Dr. Alpert of the Merck Institute for Therapeutic Research, Dr. Kenneth W. Thompson of the Schering Corporation, and Dr. Albert L. Raymond of G. D. Searle and Company.

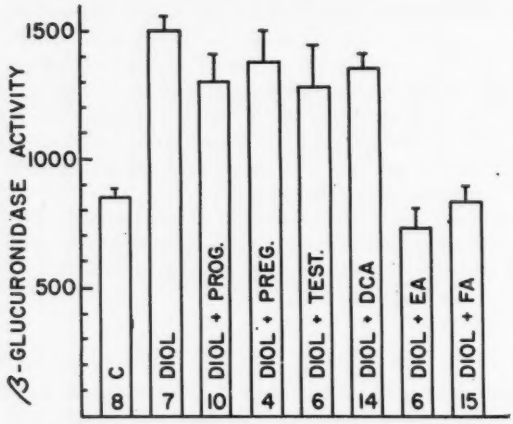

FIG. 1. The influence of crystalline steroids on perputial β-glucuronidase activity in the castrate rat. Estradiol-17β (0.5 μg. per 100 gm. body weight), was injected intravenously into all animals except group C (untreated controls). Where indicated, the additional crystalline steroids were administereed simultaneously by the intravenous route in saline suspension in the amount of 1.25 mg. per 100 gm. of body weight. Autopsy was performed 4 hours later. Abbreviations as follows: FA, Compound F-acetate; EA, cortisone acetate; DCA, desoxycortisone acetate; PREG, pregnenolone; PROG, progesterone; TEST, testosterone. Deviations shown are standard errors. The number of animals contributing to the analyses is represented by the figures at the base of the bars. β-Glucuronidase activity (cf., Talalay, Fishman and Huggins, 1946) is expressed as units per mg. wet weight of tissue. One unit of activity is that amount of enzyme which liberates one μg. of phenolphthalein per hour from the substrate phenolphthalein-mono-β-glucuronide at pH 4.5 and 38° C.

on the estrogen-stimulating effect on preputial β-glucuronidase activity.

These results suggest that many of the interactions known to occur among the various steroid hormones may be mediated through the influences which these steroids have upon certain enzyme systems. Whether the enzyme β-glucuronidase, in turn, plays as prominent a role in the mediation of estrogen activity as Fishman (1951) suggests awaits the results of further study. It is possible that preputial β-glucuronidase activity is merely a secondary manifestation of estrogenic stimulation of the reproductive tract, and may bear no direct relationship to the metabolism of the steroid hormones or to their specific metabolic effects (cf., Szego and Roberts, 1953).

Regardless of the elucidation of the precise role of β-glucuronidase in the preputial glands of the rat, these structures would appear to be ideal loci for the study of steroid hormone-β-glucuronidase interrelationships by virtue of their extremely high β-glucuronidase activity and the remarkable degree of responsiveness of this source of enzyme to steroidal influence.

SUMMARY

β-Glucuronidase activity in the preputial gland of the rat was observed to be sharply depressed 14–17 days following ovariectomy. Estrogen treatment in acute experiments effected restoration to precastration levels. A sharp distinction was observed between those adrenal steroids capable of blocking this manifestation of estrogenic activity (compounds F and E) and those closely related materials which were ineffective in this regard (DCA, progesterone, pregnenolone and testosterone). The significance of these results is discussed in the light of similar structural requirements for antagonism to estrogen activity exerted on another target organ, the uterus.

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THE EFFECT OF ACTH AND CORTISONE ON THE HYPERGLYCEMIC RESPONSE TO GLUCAGON

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DURING the course of administering glucagon (hyperglycemic-glycogenolytic factor) intravenously to rabbits it was found that in an occasional animal this factor produced an insignificant elevation or no rise in blood glucose. The site of the hyperglycemic action of glucagon appears to be the liver (Collens and Murlin, 1929); presumably, therefore, any factor that would increase the glycogen content of the liver should enhance the hyperglycemic effect of glucagon. It is well known that Cortisone will increase the liver glycogen stores. Since ACTH increases the glucocorticoid output of the adrenal glands, it should have a similar biological activity. Consequently, glucagon was administered intravenously to a group of rabbits before and after treatment with ACTH or with Cortisone.

At the time this project was started no reports could be found in the literature showing that ACTH caused an increased in the liver-glycogen content in normal rabbits. Therefore the effect of ACTH on the liver and muscle-glycogen content of normal rabbits was determined. After the completion of the experimental work for this paper, a report by Harris, Bland, Hughes, and Constable (1953) appeared which demonstrated that ACTH causes a marked increase in liver weight in guinea pigs and rabbits. The enlargement is associated with an increase in absolute amounts of glycogen, water, and protein.

MATERIALS AND METHODS

Albino rabbits weighing about 3 kg., fed Purina rabbit chow, were used. The glucagon was injected into the marginal ear vein and the variation in blood glucose was followed over a period of 90 to 120 minutes. Glucose was determined by the method of Nelson (1944) on 0.1 cc. of blood.

Two lots of glucagon were furnished by Dr. A. Staub of the Lilly Research Laboratories (225–20-233 and 208-108B-284). They were prepared by methods similar to those reported by Staub, Sinn, and Behrens (1953), but were not crystalline. The ACTH was a long-acting preparation containing 40 U.S.P. units per cc. It was injected subcutaneously in 1 cc. doses for two to three days before the second course of glucagon. Cortisone

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(Cortone, Merck) was given for three days subcutaneously (10 mg. in the morning and 10 mg. in the evening).

For the determination of the effect of ACTH on liver glycogen, female albino rabbits, all of the same strain, were used. The animals were maintained, two to a cage, in an air-conditioned room, on a diet of rabbit pellets and water *ad libitum*. After two or three days, one rabbit in each cage was treated with ACTH, 1.0 ml. administered intramuscularly or subcutaneously once a day for three days. The untreated cage mate served as a control. On the fourth day all of the rabbits were anesthetized with "Sodium Seconal" (*Secobarbital Sodium, Lilly*) intravenously. Pieces of liver were quickly removed and placed in hot 30% KOH solution for glycogen determination by the method of Good, Kramer, and Somogyi (1933). Pieces of muscle from each hind leg were also taken for glycogen determination.

RESULTS

Influence of ACTH and Cortisone on the repsonse of blood sugar to glucagon. The effect of pretreatment with ACTH on the elevation of blood glucose of rabbits after the intravenous administration of glucagon is shown in Table 1. The elevation of blood glucose in response to glucagon was significantly greater after ACTH treatment. In addition, the area under the curve was enlarged (Fig. 1, left panel).

After ACTH, only two animals out of 23 gave less than a 20 mg.% rise in blood glucose in response to glucagon. Without ACTH treatment, in 15 animals the blood glucose rise was 20 mg.% or less, six animals showing 10 mg.% or less elevation.

In several of the rabbits not treated with ACTH, doubling the dose of glucagon did not cause a greater rise in blood glucose. For example, a rabbit given 15 μg. of lot 225-20-33 per kg. had an elevation of blood glucose of 17 mg.%; with 30 μg. per kg., there was a rise of 18 mg.%. After ACTH treatment, 15 μg. per kg. of glucagon gave a 35 mg.% glucose elevation and 30 μg. per kg. raised the blood glucose 52 mg.%. When a

TABLE 1. MAXIMUM CHANGE IN BLOOD SUGAR (MG. %) OF RABBITS GIVEN GLUCAGON INTRAVENOUSLY BEFORE AND AFTER ACTH TREATMENT

Lot 225-20-233—15μg./kg.			Lot 225-20-233—30 μg./kg.			Lot 208-108B-284—1 μg./kg.		
Before ACTH	After ACTH	Change	Before ACTH	After ACTH	Change	Before ACTH	After ACTH	Change
20	38	18	25	47	22	24	28	4
28	57	29	18	52	34	13	26	13
1	37	36	32	36	4	4	23	19
24	28	4	−1	102	103	23	25	2
17	35	18				20	15	−5
23	27	4				3	22	19
10	26	16				13	25	12
18	31	13				16	15	−1
25	45	20				16	25	9
						4	27	23
18.4±2.8	36±3.33	17.6±3.54*	18.5±7.1	59.3±14.6	40.8±21.65†	13.6±2.46	23.1±1.50	9.5±2.94‡

* P = <0.01
† P = 0.05
‡ P = 0.01

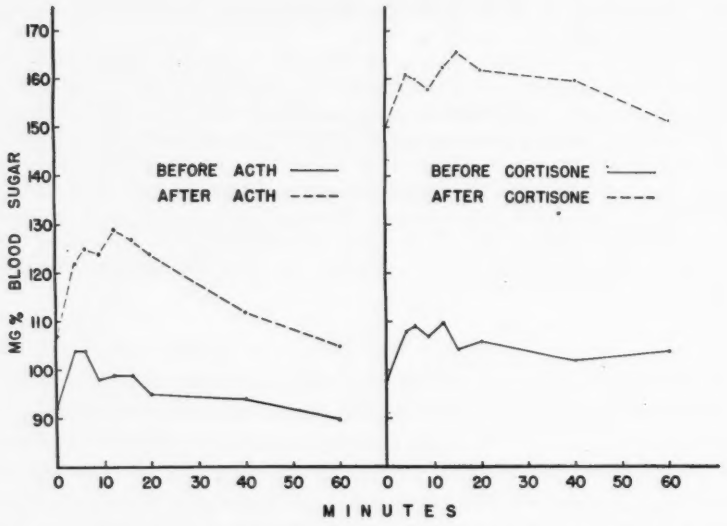

FIG. 1. Increase in blood sugar in rabbits following intravenous injection of glucagon.
Left panel: Average blood sugar curve of ten rabbits given glucagon, Lot 208-108B-284 (1.0 μg. per kg.) before and after ACTH treatment (3 days).
Right panel: Average blood sugar curve of four rabbits given glucagon, Lot 225-20-233 (15 μg. per kg.) before and after Cortisone treatment (3 days).

series of ACTH-treated rabbits were injected with 0.5 μg., 1.0 μg., and 1.5 μg. lot 208-108B-284 per kg., the average increase in blood glucose of six rabbits at the 0.5 μg. level was 16 mg.%, of 21 rabbits at the 1.0 μg. level was 28 mg.%, and of six rabbits at the 1.5 μg. level was 38 mg.%.

ACTH had a moderate diabetogenic effect. The control blood glucose levels before ACTH treatment for all of the rabbits averaged 94 mg.% with a range of 69 to 116 mg.%. After ACTH, the average control glucose level was 112 mg.% with a range of 89 to 142 mg.%.

The effect of Cortisone on the response to glucagon was quite different from that of ACTH (Fig. 1, right panel). The elevation of blood glucose after Cortisone treatment was not significantly greater than before treatment; however, Cortisone showed a marked diabetogenic effect. Before Cortisone administration the control glucose levels averaged 97.5 mg.% with a range of 75 to 105 mg.%. After Cortisone, the controls averaged 150 mg.%, with a range of 126 to 175 mg.%.

The effect of ACTH on liver and muscle glycogen. Two separate experiments were carried out using the same methods except that in the first one the ACTH was administered intramuscularly and in the second it was injected subcutaneously. The results obtained in the two experiments

TABLE 2. EFFECT OF ACTH ADMINISTRATION ON GLYCOGEN OF LIVER AND MUSCLE

	Control			ACTH treated			
	I	II	Average I & II	I	II	Average I & II	P*
Body weight (kg.)	1.579	2.024	1.801	1.652	2.118	1.885	
Liver weight (gm.)	52.53	64.52	58.53	74.72	89.92	84.02	
Liver glycogen (gm./100 gm.)	6.28	4.41	5.18	6.44	5.51	5.97	
Liver glycogen (gm. total)	3.41±1.80	2.56±2.34	2.98±1.92	5.34±3.24	5.28±3.66	5.31±3.12	<0.01
Muscle glycogen (mg./gm.)	560.0	604.0	584.0±180.0	753.0	825.0	789.0±191.0	<0.01
Liver water (gm./100 gm.)		72.2			67.4		
Liver water (gm. total)		46.6±2.96			61.1±18.7		<0.01

* Calculated on the basis of the combined values of experiments I and II.

were similar enough so that the figures have been pooled and treated as one. Table 2 gives the data for the individual experiments as well as the combined results. The significance of the differences between treated and control animals was calculated on the basis of the combined figures.

It is apparent that the administration of a long-acting preparation of ACTH for three days increased the glycogen stores in both liver and muscle. The change in muscle glycogen consisted of an increase in concentration, whereas the change in liver glycogen was not in the concentration but in total content. This increase in total glycogen content of the liver is reflected in, or is caused by, a marked increase in liver weight in the treated animals.

In the second experiment, in order to determine water content, pieces of liver were dried to constant weight in an oven at 100° C. The concentration of water was higher in the control animals than in those treated with ACTH, but the total liver water was significantly greater in the treated rabbits. The greater weight of glycogen and water in the livers of treated animals is not sufficient to account for the total increase in liver weight in these animals. About 65% of the change in weight can be accounted for by glycogen and water. Whether the remaining 35% is composed of protein, fat, or both, has not been determined.

DISCUSSION

The data presented in this paper and in that of Harris *et al.* (1953) show that ACTH caused an increase in the glycogen content of the liver of rabbits, and a marked increase in liver weight was found by both groups. The gain in weight can be accounted for by increases in water, glycogen, and protein. Harris *et al.* (1953) feel that the action of ACTH on the liver can be attributed to its influence on stimulating the production of Cortisone.

The administration of long-acting ACTH to rabbits enhanced the hyper-

glycemic response to glucagon. It is possible that this effect might be due to a higher content of glycogen in the liver. However, other hormonal actions of ACTH might be responsible, particularly since after Cortisone, which also increases liver glycogen in rabbits (Harris *et al.*, 1953; Kerrpola, 1952), glucagon did not cause a greater elevation of blood sugar.

The diabetogenic effect of Cortisone was found to be quite marked—greater than that of ACTH. The high control blood sugar values after Cortisone might have accounted for the failure of glucagon to show an enhanced action. It is interesting to note that Kerrpola did not find that epinephrine caused a greater rise in blood glucose in rabbits that had been treated with Cortisone. He, too, reported a marked elevation in control blood sugar values following Cortisone administration.

After a course of ACTH, glucagon caused an elevation in blood glucose in every rabbit, whereas before treatment several animals failed to respond or showed a poor response to the doses given. Untreated rabbits sometimes did not show graded responses to increasing doses of glucagon. After ACTH it was possible to distinguish between doses of glucagon varying over a fairly wide range. Rabbits treated with long-acting ACTH might prove to be good test animals for glucagon assays.

SUMMARY

The administration of long-acting ACTH to rabbits enhanced the hyperglycemic response to glucagon.

Glucagon did not have an increased effect in elevating blood glucose in rabbits that had been treated with Cortisone.

Cortisone caused a much greater elevation in the control blood sugar values than ACTH.

A marked increase was found in the liver weight of rabbits treated with long-acting ACTH. About 65% of this change in weight could be attributed to glycogen and water.

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THE FATE OF CORTISONE-*T* IN MICE[1]

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KNOWLEDGE of the tissue distribution, the chemical transformation which the molecule undergoes and the eventual disposal of the end products of hormone metabolism may afford significant information with respect to the mode of action or the site of effectiveness of these agents. Certain initial investigations of this problem dealing with the localization and fate of radioactive testosterone in rodents have been reported from this laboratory (Barry, Eidinoff, Dobriner and Gallagher, 1952). In continuation of these investigations, we have studied the tissue distribution of an adrenal hormone, cortisone labeled with tritium and with the greater portion of the isotope in chemically stable locations in the molecule (Fukushima, Kritchevsky, Eidinoff and Gallagher, 1952). We have examined as well the route of elimination of the hormone or its metabolic end products.

A series of orienting experiments were carried out in a semi-quantitative fashion where the counting errors were large. These preliminary studies afforded a perspective of the principal avenues of distribution of the hormone and its derivatives as well as the route and amount of the excretory product. The salient findings from these orientation studies were then repeated in more critical experiments where the counting was done with an acceptable degree of precision. This report describes the details of the quantitative studies dealing with the localization and excretion in mice of cortisone, administered by several methods.

EXPERIMENTAL

Isotopic analysis.—The cortisone-*t*, when analyzed by the procedure described elsewhere (Fukushima, Kritchevsky, Eidinoff and Gallagher, 1952) had a specific activity of 1030 microcuries per millimole; when spread

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as an infinitely thin film on steel planchets the material had a specific activity of 2.25×10^6 cts./min./mg. using a Tracerlab SC-16 flow gas counter connected to a Tracerlab 64 scaler. The cortisone alcohol was dissolved in 1:9 propylene glycol-water (0.3–0.4 mg./ml.) or in physiological saline. From 20–80 micrograms were injected in the various experiments. Preliminary experiments demonstrated that while direct plating permitted one to detect the presence of tritium in biological extracts, this counting procedure was not satisfactory for quantitative work with extracts of low specific activity. The problem arises from the low surface density (0.7–0.8 mg./cm.^{2}) of an infinitely thick layer for tritium, a value that should be compared with the 25–30 mg./cm.^{2} for an infinitely thick layer of C^{14} or S^{35}. Attempts to use extracts adjusted to the same concentration of solids with any of several different substances failed to yield reproducible plates as the result of partial segregation of the components during drying of the samples. In addition, the count per plate obtained from a given amount of tritium labeled material was found to vary by 10–15 per cent with the addition of different substances. The use of infinitely thin samples was precluded by the requirement of surface densities below 5 μg./cm.^{2} (the upper limit for infinitely thin samples); for many tissue extracts this necessitated such dilution that the count per plate was below an accurate figure. Thus a 100 mg. sample with 15,000 c/m would require a dilution of 1:2000 to bring the concentration of solids per ml. to the 50 μg. limit for 10 cm.^{2} planchets. This dilution would reduce the count per plate to 7 cts./min./plate above background.

Heard, Thompson, and Yates (1952) have described an isotopic dilution technique whereby these problems can be circumvented. The scheme involves diluting known amounts of the extract to be counted with two different known concentrations of a radioactive substance of high specific activity (materials I and II) and counting the resultant product. From the activity of these mixtures of extracts of unknown activity and of known activity, one can compute the activity the extract would have if it were counted at infinite thinness by the following equation for volumetric analysis:

$$\text{True Ct. per Ml. of Extract} = \frac{x(N_2S_1 - N_1S_2)}{A(N_1 - N_2)}$$

where x = Vol. of materials I and II employed.
A = Vol. of the aliquot of the extract to be analyzed.
S_1 = S.A. (cts./min./ml.) of material I added to the first aliquot.
S_2 = S.A. (cts./min./ml.) of material II added to the second aliquot.
N_1 = Average ct. of the plates made from the first mixture.
N_2 = Average ct. of the plates prepared from the second mixture.

The method has proved to be relatively satisfactory for assaying biological extracts containing tritium at various activity levels. The chief difficulty lies in the extremely high accuracy required for the values of N_1 and N_2. Depending upon the particular values of S_1 and S_2 employed, an error of 1 per cent in either N_1 or N_2 can cause a 5–10 per cent error in the true activity value.

General procedure.—In all except the very short term experiments the mice were kept in a glass cage which separated the urine and the feces. In experiments lasting less than 6 hours, the mice were given only water while in longer experiments they were also given food *ad libitum*. At the end of the experimental period, the mice were anesthetized, killed by bleeding and the various tissues separated. Since there was usually urine in the bladder, the contents were pooled with the excreted urine. The specimens were dried under reduced pressure at room temperature, powdered in a mortar with sand or sodium sulfate, and continuously extracted with alcohol for 36–48 hours. The extracts were concentrated, diluted to volume and analyzed by the isotopic dilution procedure. When necessary, extracts were partitioned between chloroform and water and the two fractions were separately analyzed.

Intravenous series (*Table 1*).—In these experiments the cortisone in propylene glycol-water was injected into the tail vein of unanesthetized mice held in a restraining cage. In the 5-minute experiment, the mouse was rapidly anesthetized 5 minutes after the injection and then bled from the blood vessels of the shoulder to collect a blood sample. This procedure required about 2–3 minutes. The mouse was then dissected and the various organs removed. The total procedure from injection to death took about 8 minutes. The various tissues were worked up as described above. The two 2-hour experiments were carried out in the same fashion.

In the 24-hour experiment, the urine was collected in three portions and the feces in two portions which were individually analyzed. The values for the urine were: 0–3 hours, 24,500 cts./min. (33.0% of the dose given); 3–6 hours, 12,300 cts./min. (16.6% of the dose given); 6–24 hours, 11,700 cts./min. (15.8% of the dose given). The fecal values were: 0–6 hours, 7,900 cts./min. (10.7% of the dose given); 6–24 hours, 15,000 cts./min. (20.3% of the dose given).

Intramuscular and intraperitoneal experiments (*Table 2*).—In the intramuscular experiments, the propylene glycol-water solution was injected in the fleshy part of the hind leg. In the one hour experiment, the hind leg injected was analyzed separately. In the 24-hour experiment, three urine and three fecal collections were made. The various urine portions contained: 0–6 hours, 32,000 cts./min. (29.4% of the dose given); 6–18 hours, 6,000 cts./min. (5.5% of the dose given); 18–24 hours, 5,000 cts./min.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY AFTER THE INTRAVENOUS INJECTION OF CORTISONE IN NORMAL AND ADRENALECTOMIZED MICE

	Normal mice						Adrenalectomized mouse	
Duration of experiment	5 minutes		2 hours		24 hours		2 hours	
Dose administered	38,100 c/m 17γ		89,000 c/m 40γ		74,000 c/m 33γ		87,000 c/m 39γ	
Specimen	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose
Urine	Trace	<0.5	21,600	24.2	48,500	65.4	16,200	18.4
Feces	No sample	—	100	<0.5	22,900	31.0	No sample	—
Liver	27,200	71.5	28,400	31.9	—	—	24,000	27.6
Small Intestine	8,800	23.2	14,300	16.1	—	—	54,000	62.0
Colon			24,900	28.0	—	—	0	0
Other Viscera	Trace	<0.5	2,200	2.5	—	—	0	0
Carcass	4,100[a]	11.2	0	0	0	0	0	0
Blood	Trace	<0.5	—	—	—	—	—	—
Total	40,100	106	91,500	103	71,400	96.4	94,200	109.0

[a] The skin and the rest of the carcass were extracted separately in this experiment. Each extract was divided into chloroform soluble and water soluble fractions. For the skin extract the activity in the chloroform-soluble fraction was 1,800 c/m (4.7% of the total dose); there was only a trace of activity (<0.5%) in the water-soluble fraction. For the rest of the carcass the chloroform-soluble fraction had an activity of 2,300 c/m (6.5% of the total dose); there was no activity in the water-soluble fraction.

(4.6% of the dose given). The fecal extracts contained: 0–6 hours, 20,500 cts./min. (18.8% of the dose given); 6–18 hours, 2,000 cts./min. (1.8% of the dose given); 18–24 hours, 48,000 cts./min. (44.0% of the dose given). In the six hour intraperitoneal experiment, the value given for the liver includes the activity found in the gall bladder which was separately analyzed; 3,300 cts./min. (1.9% of the dose given).

Three minutes series (Table 3).—The bile duct ligation was carried out under ether anesthesia. The abdomen was opened and the bile duct exposed. The duct was doubly ligated with silk thread and the abdomen was then sutured. As soon as the animal revived, it was injected in the tail vein with cortisone-*t* in saline solution. After 3 minutes, the animal was rapidly anesthetized and then decapitated. Blood was collected from the stump and then the mouse was rapidly dissected. The total time required from injection to decapitation was between 3 and 3¼ minutes. A number of tissues were further subdivided for analysis. The gall bladder contained 500 cts./min. (0.5% of the dose injected) which is included in the value for the liver. The intestine was slit open and the contents washed out with a stream of water and separately assayed. The contents proved to be devoid of activity. In this experiment the head, lungs and tail were not

TABLE 2. DISTRIBUTION OF RADIOACTIVITY AFTER INTRAMUSCULAR AND INTRAPERITONEAL INJECTION OF CORTISONE IN NORMAL MICE

Site injected	Intramuscular				Intraperitoneal			
Duration of experiment	1 hour		24 hours		2 hours[a]		6 hours	
Dose administered	101,000 c/m 45γ		109,000 c/m 48γ		124,000 c/m 55γ		178,000 c/m 79γ	
Specimen	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose
Urine	7,100	7.0	43,000	39.5	17,500	14.1	18,000	10.1
Feces	0	0	70,500	64.6	1,300	1.0	36,000	20.2
Liver	26,300	26.0			40,000	32.3	16,000	9.0
Small Intestine	25,800	25.6	0	0	26,400	21.2	18,000	10.1
Colon	5,100	5.1			35,800[b]	28.9	75,000	42.1
Other Viscera	9,400	9.3	—	—	0	0	2,800	1.6
Carcass	0	0	—	—	0	0	—	—
Blood	Trace	<0.5	—	—	—	—	—	—
Site of Injection	17,300	17.1	—	—	—	—	—	—
Total	91,000	90.1	113,000	104.1	121,000	97.5	166,000	93.0

[a] In this experiment two mice were employed. Each received half of the injection. The appropriate extracts were combined.

[b] The colon extract was partitioned between chloroform and water. The chloroform extract contained 34,400 c/m (27.8% of the dose), the water extract contained 1,400 c/m (1.1% of the dose given).

separated from the rest of the carcass, in the other experiments of this series they were analyzed separately.

A sham operation was carried out on another mouse. The abdomen was opened and the gut manipulated as for a bile duct ligation. The animal was then sutured and just permitted to revive before the injection (164,000

TABLE 3. DISTRIBUTION OF ACTIVITY THREE MINUTES AFTER THE INTRAVENOUS ADMINISTRATION OF CORTISONE TO OPERATED AND UNOPERATED MICE

	Ligated bile duct		Anesthesia, no surgery		No anesthesia, no surgery	
Dose administered	98,000 c/m 44γ		113,000 c/m 50γ		120,000 c/m 54γ	
Specimen	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose	Total cts/min.	Per cent of dose
Liver	8,500	8.7	41,000	36.0	32,000	26.6
Blood	7,000	7.2	1,400	1.2	10,000	8.3
Small Intestine	3,100	3.2	9,600	8.5	3,500	2.9
Colon	1,800	1.8	2,000	1.8	0	0
Other Viscera	0	0	1,900	1.7	2,100	1.7
Carcass	79,000	81	35,400	31.7	81,300	67.6
Skin	3,700	3.8	11,000	9.7	9,300	7.7
Tail	—	—	1,600	1.4	0	0
Total	103,000	106	104,000	91.8	138,000	114.8

cts./min.) was given. Unfortunately, a large part of the injection solution (111,000 cts./min.) entered the vein canal and was found in the tail at the end of the experiment. Assuming the remainder of the radioactivity (53,000 cts./min. circulated through the body of the mouse, the following distribution was obtained: liver, 5,500 cts./min. (9.7%); blood, none; small intestine, 3,600 cts./min. (6.7%); colon, none; other viscera, none; carcass, 43,000 cts./min. (81%); and skin, 1,900 cts./min. (3.6%). These results are quite similar to those found.in the bile duct ligation experiment.

In the anesthesia control experiment, the mouse was anesthetized as for surgery and permitted to revive before injecting the cortisone. The rest of manipulative details were as described above. In this case, the combined duodenum and jejunum and the ileum were separately analyzed. The duodenum plus jejunum contained 7,000 cts./min. (6.2% of the dose given); the ileum contained 2,600 cts./min. (2.3% of the dose given). The value for the carcass includes the separate values for the head and the lungs which contained 3,100 cts./min. (2.7% of the dose given) and 2,300 cts./min. (2.0% of the dose given) respectively.

Except for the absence of pretreatment, the control experiment was carried out as described above. The combined duodenum plus jejunum contained 3,500 cts./min. (2.9% of the dose given) while the ileum was inactive. The activity in the carcass includes that in the head and the lungs which contained 9,700 cts./min. (8.1% of the dose given) and 600 cts./min. (0.5% of the dose given) respectively.

The results obtained from the short term experiment of Table 1 and the control experiment of Table 3 are somewhat different. A plausible explanation is that the higher concentration of radioactivity in the liver in the first experiment reflects the longer time of circulation (approximately 8 minutes) as compared with a shorter term (3 minutes) in the second experiment. The difference observed is then a result of the time required for the radioactive metabolites to accumulate in the liver. Another difference was in the solvent employed in the two experiments. In the first, propylene glycol-water was used, in the second, physiological saline. Since an extended series would be required to settle the problem, no real explanation can be advanced at this time for the observation reported.

DISCUSSION

These studies show that cortisone, regardless of the route administered, resides briefly in tissues, reaching for a short time a significantly high concentration in the liver, and gastrointestinal tract and a very much smaller concentration in skin and carcass. Indeed, within 3 to 8 minutes after an intravenous dose, very large amounts of the radioactivity injected were in the liver and gut. These experiments lend support to the view that the localization of cortisone in the liver is specific for that molecule and is

not a property shared with the immediate metabolites and degradation products of the hormone. Much of the hormone or its metabolites rapidly reaches the gastrointestinal tract and yet so large a portion is eventually excreted in urine, it is evident that reabsorption of this material must occur in the longer term experiments. A similar result was recorded for the metabolites of testosterone in rodents (Barry, Eidinoff, Dobriner and Gallagher, 1952). Since the radioactivity found in the liver steadily diminishes with time after administration, it appears that reabsorbed metabolites of the hormone are not retained in the liver in the same selective manner exhibited with the hormone itself.

After injection of cortisone intravenously, a considerably greater amount of radioactivity was excreted in the urine than when the hormone was given intramuscularly or intraperitoneally. That this result reflects a real difference in metabolic pathway and not simply rapid clearance of excessive hormone by the kidney is evidenced by the fact that the excretion was not immediate but gradually increased with time (5 minutes = less than 0.5% of dose; 2 hours = 24% of dose; 65% of dose after 24 hours). The difference between the intravenous and other routes may be the result of alteration of part of the hormone locally at the injection site, to differences in rate of absorption, to excretion in the gut followed by reabsorption and recirculation or to many other factors, the significance of which is not clear at this time. Since the hormones normally must be secreted into the blood, it seems reasonable to suggest that the results obtained after introduction of the hormone by vein more nearly mirror physiological events.

In the normal mouse and in the adrenalectomized animal there was little difference in the tissue distribution and the ultimate fate of the hormone. This similarity of localization and metabolism of cortisone in the animal having an adequate hormone supply provides no support for the concept of a "tissue demand" leading to an increased retention of the hormone in tissues, a different localization or an altered metabolism of any major fraction of the administered hormone. The view may be taken, of course, that only the minute amount of the administered hormone residing in the peripheral tissues for a measurable interval is an effective agent. As a consequence, the large amount of hormone that must be administered to demonstrate a physiological effect is required only in order to produce sufficient concentration in the body fluids to enable the receptor organs to remove from circulation the relatively few molecules of active material necessary to regulate the biochemical processes under hormonal control. If this be the case, it is evident that far more sensitive measurements than determination of radioactivity of whole organs must be undertaken to explore this possibility.

The mode of action of the steroid hormones could be related to a type of

"trigger mechanism" i.e., a brief encounter of tissue with hormone followed by a chain of reactions which, once initiated, results in a dynamic series of biochemical events. These might be envisioned as mediating or regulating the synthesis of enzymes or of prosthetic groups. Or, alternatively, a portion might be combined with protein and this complex might be the functional hormone. Viewed in the light of the hypothesis that a major portion of the available hormone must localize in tissues for a time interval of measurable duration in order to exert its physiological function, the results reported would point to the liver and gut of rodents as the principal receptor organs for cortisone. This concept could be expanded to include the other steroid hormones as well, since a similar localization has been demonstrated for both testosterone (Barry, Eidinoff, Dobriner and Gallagher, 1952) and progesterone (Barry, Eidinoff, Dobriner and Gallagher, 1952; Riegel, Hartop and Kittinger, 1950). Both testosterone and cortisone have, in the broad sense, a profound effect upon the metabolic processes of the organism and in particular, upon the biochemical mechanisms involved in the synthesis, degradation and transfer of protein. Because of the essential role of the liver in protein metabolism, it is quite reasonable to suppose that the steroid hormones quite markedly affect the metabolism of that organ and might therefore be retained in very much higher amount than in other tissues. It may well be that the hormonal regulation of protein metabolism is accomplished chiefly in the liver, and that the changes in peripheral tissues are dependent upon and secondary to this primary and obligatory action of the hormone in the liver. According to this viewpoint, the hormones are selectively concentrated in the tissues whose metabolic processes they regulate or control. It was earlier suggested by us (Barry, Eidinoff, Dobriner and Gallagher, 1952) that the accumulation of radioactivity in the liver following the administration of testosterone and progesterone reflected a purely excretory function of that organ, concerned only with the elimination of the hormone from the body. While this suggestion may not be completely disregarded, the alternate possibility that the site of principal effectiveness of the hormone is the liver and gastro-intestinal tract must certainly be given serious consideration.

These studies reinforce the conclusion affirmed in our earlier report that the residence of the steroid hormones in tissues is remarkably short. During this brief time, the molecule undergoes biochemical transformation and finally the metabolites are excreted. Almost identical distribution patterns were observed when cortisone was administered intravenously to normal and adrenalectomized mice. It was previously observed that when testosterone was administered to the castrate, the intact male, and the female mouse, the distribution pattern was quite similar. It therefore seems

improbable that tissue of normal animals is saturated with hormones and/or metabolites so that administered hormone is treated as excess of need and in consequence is rapidly eliminated. The experiments reported support rather the view that the biochemical time span of cortisone and testosterone *as hormones*, is remarkably short and is consistent with the view that they function by either a trigger mechanism or as components of some system in the liver where localization has been demonstrated.

Finally it is apparent that very little of the administered mateiral could have been completely metabolized with loss of the isotopic label since recovery of the total number of counts administered was essentially quantitative. Since much of the radioactivity was at C-16 of the cortisone molecule, this complete recovery proves that no great alteration in this portion of the hormone had taken place. It is quite likely although it has not been studied, that the type of metabolite formed by the rodent and man are not the same, a difference strikingly apparent with the metabolites of testosterone in the two species. Studies of such basic differences nevertheless require further elaboration and should afford an important contribution to the comparative biochemistry of the steroid hormones.

SUMMARY

After the administration of tritium labeled cortisone to mice, by intraperitoneal, intramuscular and intravenous injection, radioactivity was excreted in urine and feces. The recovery of the isotope was essentially quantitative. A greater portion of the total radioactivity excreted was found in the feces after intraperitoneal and intramuscular injection; after intravenous injection, the greater portion of the radioactivity was excreted in the urine. No major destruction of the ring system of cortisone occurred during the time intervals studied. At very short time intervals after intravenous administration, appreciable amounts of radioactivity were found through the entire carcass. At longer times this material was found in the liver and after one hour most of the radiaoctivity was present in the urine, feces and gastro-intestinal tract. Evidence was obtained that a portion of the radioactivity was excreted in the bile. There was reabsorption of radioactivity from the intestine and this material was then in part excreted in the urine.

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NOTES AND COMMENTS

THE HYDROLYSIS OF PREGNANEDIOL GLUCURONIDE BY GLUCURONIDASE PREPARATIONS[1]

We have recently published an assay technique for pregnanediol using glucuronidase (Goldfine and Cohen, 1953) for the hydrolysis of pregnanediol glucuronide. A year and a half ago Dr. H. Guterman reported to us some difficulties he was having with the VioBin glucuronidase. We were therefore led to study this preparation of glucuronidase. Indeed, we were not able to obtain more than about 50% hydrolysis of sodium pregnanediol glucuronidate with 1000 units (Fishman) of this preparation. We have, therefore, investigated the hydrolyzing powers of a number of commercial preparations.[2] The corticoids and ketosteroids were hydrolyzed for 4 days with 10,000 Fishman units per 50 cc. of urine and assayed according to the procedures of Cohen (1951). Pregnanediol hydrolysis and assays were carried out by the technique of Goldfine and Cohen (1953).

The results are shown in Tables 1 and 2. It is obvious from these results that all preparations seem to have an equal capacity on corticoid and ketosteroid glucuronide hydrolysis. However, the dry preparation had a limited effect on the hydrolysis of pregnanediol glucuronide. It is readily seen, however, that only our own and the Warner preparation give an acceptable hydrolysis of pregnanediol glucuronide. The use of 5000 units gave somewhat better, but still not accurate, results. Bacterial hydrolysis was of little value in our tests, since 1000 and 5000 units both liberated ketosteroids completely

TABLE 1. HYDROLYSIS OF KETOSTEROID AND CORTICOID GLUCURONIDES

Enzyme prep.	Urine	mg. Ks/L.	Urine	mg. Ks/L.
Glucuronidase 59	NMU37*	6.7	NMU39	6.0
VioBin I	NMU37*	6.3	NMU39	5.0
VioBin II	NMU37*	6.1	NMU39	5.7
Tec I	NMU37*	6.1	NMU39	5.3
Tec II	NMU37*	6.4	NMU39	
Mann Fine	NMU37*	7.1	NMU39	5.2
Warner	NMU37*	7.2	NMU39	5.6
Average		6.6± .6		5.4±0.6
Enzyme prep.	**Urine**	**mg. corticoid/L.**	**Urine**	**mg. corticoid/L.**
Glucuronidase 59	NMU35	11.9	NMU37	23.6
VioBin I	NMU35	12.5	NMU37	27.0
VioBin II	NMU35	12.4	NMU37	29.8
Tec I	NMU35	12.8	NMU37	22.6
Tec II	NMU35	12.4		
Mann Fine	NMU35	12.8	NMU37	24.6
Warner	NMU35	11.9	NMU37	23.6
Average		12.4±0.5		25.2±4.6

* NMU refers to unknown normal male urine, batch 37, etc.

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TABLE 2. RELEASE OF PREGNANEDIOL FROM URINE BY VARIOUS ENZYME PREPARATIONS

Amount Pregnanediol present	Amount of glucuronidase	Glucuronidase preparation	Amount pregnanediol released	Pregnanediol released
			mg.	%
1.64 mg. as pure NaPG	1000 units	Glucuronidase 51	1.6	100
	1000 units	VioBin I	0.78	48
	1000 units	VioBin II	0.90	55
			mg./l	
Urine Batch LPU—C_1*	1000 units	Glucuronidase 53	29.0	
	1000 units	VioBin II	11	40
	1000 units	Tec (25,000 units/gm.)	13.2	46
	1000 units	Bact.	7.6	26
LPU—C_2	1000 units	Glucuronidase 58	39.1	
	1000 units	VioBin III	10.3	26
	1000 units	Tec I (25,000 units/gm.)	11.5	29
	1000 units	Tec II (90,000 units/gm.)	11.4	29
	1000 units	Mann (25,000 units/gm.)	9.2	24
	1000 units	Warner 1 (5000 units/cc)	40.1	102
LPU—C_3	1000 units	Glucuronidase 58	20.0	
	5000 units	VioBin III	15.7	79
	5000 units	Tec I	13.0	65
	5000 units	Tec II	15.7	79
	5000 units	Mann	18.0	90
	5000 units	Warner 1	21.7	107
LPU—C_4	1000 units	Glucuronidase 58	23.8	
	1000 units	Warner 2	24.1	104
	1000 units	Bact. 2	4.3	18
	5000 units	Bact. 2	21.4	90
NFU (Normal Female Urine)	1000 units	Glucuronidase 58	2.44	
	1000 units	Warner 2	2.46	100
	1000 units	Warner 3	2.47	101

* LPU equals late pregnancy urine, batch C_1, C_2, etc.

[2] The enzyme preparations used were obtained as follows:

The VioBin preparations were obtained from the VioBin Corporation, Monticello, Illinois. Three VioBin preparations were tested (I, II, and III each containing 25,000 units per gram).

The Tec preparations were obtained from the Tec Chemical Corporation, Stamford, Connecticut. Two preparations, one containing 25,000 units per gram (I) and one containing 90,000 units per gram (II), were available.

The Mann Preparation was obtained from the Mann Research Laboratories, Inc., New York, N. Y. and contained 25,000 units per gram.

The Warner Preparation was obtained from the Warner-Chilcott Laboratories, New York, N. Y. This is a preparation of beta glucuronidase prepared from beef liver and contained 5000 units per cc.

All of these enzyme preparations were solids except the last named. Our own preparation of glucuronidase was prepared from spleen (Cohen, 1951) kindly made available to us by the VioBin Corporation.

(Buehler *et al.*, 1949), whereas the release of pregnanediol was incomplete with 1000 units (see Table 2).

Recently the VioBin Corporation sent us some glucuronidase solution, which we have tested and found to hydrolyze pregnanediol glucuronide completely.

DISCUSSION AND SUMMARY

The reason why the dry preparations do not give quantitative yields of pregnanediol remains obscure. The fact that enzyme preparations will hydrolyze ketosteroid and corticoid glucuronides completely while having only a limited effect on pregnanediol glucuronide, indicates the complexity of the problem. It is possible, of course, that a number of different glucuronidases are present in this preparation, some more specific than others. It is also possible that some coenzyme for glucuronidase action may be destroyed during the drying process (Bernfeld and Fishman, 1953).

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HISTOCHEMICAL DEMONSTRATION OF PROTEIN-BOUND SULFHYDRYL AND DISULFIDE GROUPS IN CELLS OF THE ANTERIOR PITUITARY[1]

The introduction of recent histochemical techniques has stimulated a new interest in the morphology and the histophysiology of the anterior pituitary. Reports have appeared on a variety of reactions and substances demonstrable in the anterior pituitary gland by histochemical means. These include the periodic-acid Schiff (PAS) reaction (Catchpole, 1949; Pearse, 1948, 1949, 1950, 1952, 1953; Purves and Griesbach, 1951), acid phosphatase activity (Abolins, 1948; Abolins and Abolins, 1949), the presence of phospholipids (Finerty, Hess and Binhammer 1952; Rennels, 1953), cytoplasmic basophilia (Desclin, 1940, 1945; Dempsey and Wislocki, 1945; Wolfe, 1949), metachromasia (Herlant, 1943), the presence of ascorbic acid (Mancini and de Pasqualini, 1950 a, b, c) and the method using labeled antibodies (Marshall, 1951).

Histochemical methods for demonstrating protein-bound sulfhydryl and disulfide

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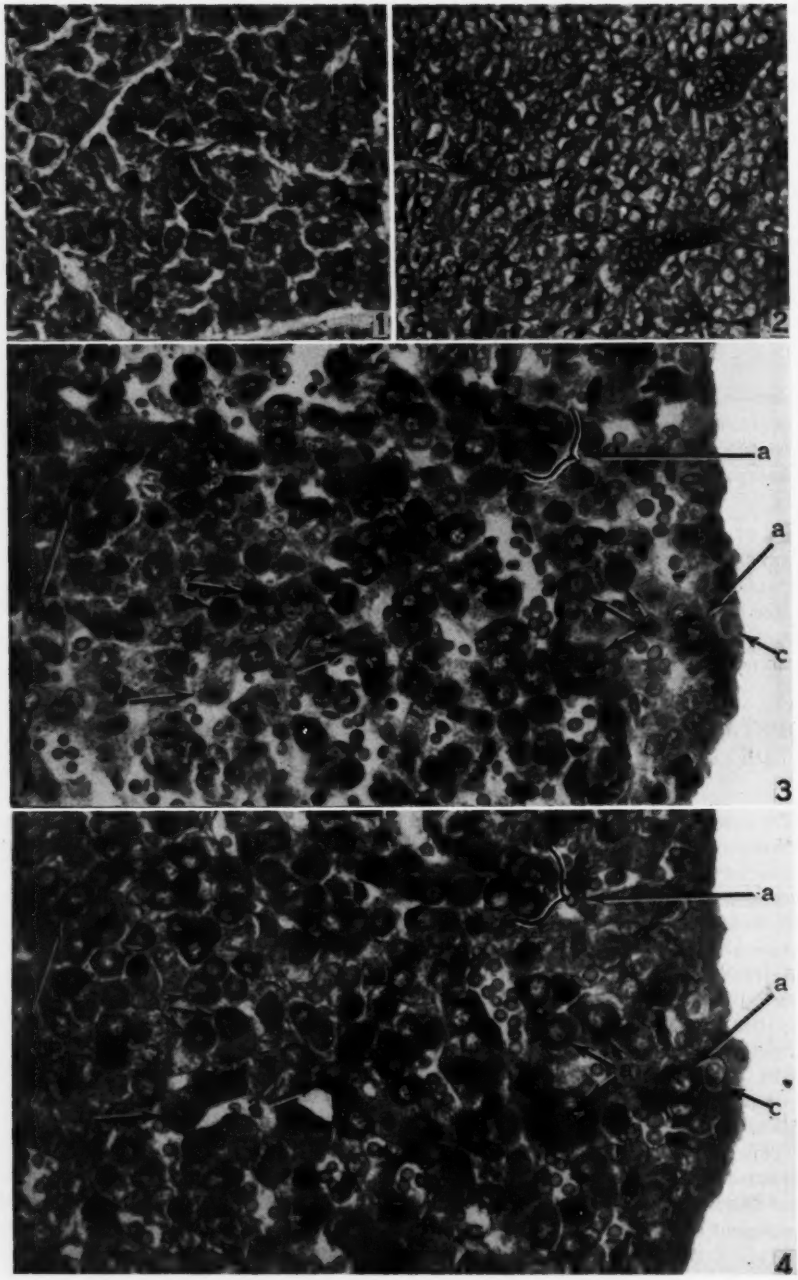

FIGS. 1–4

groups (Barrnett and Seligman, 1954a) have recently been developed based upon a specific technique for staining sulhydryl groups (Barrnett and Seligman, 1952; Barrnett, 1953a). In the present paper we shall show that sulfhydryl and/or disulfide groups are localized in the cytoplasm of anterior pituitary cells that have been called "acidophiles."

MATERIALS AND METHODS

The pituitary glands of normal adult mice and rats of both sexes constituted the principal material, but a few observations were also made on dog, cat, and monkey. The pituitary glands were fixed in Zenker's fluid, or in a solution of 1% trichloracetic acid in 80% ethyl alcohol (80-TCA). In a few instances, 10% formalin, sublimate-formol (9 parts saturated aqueous solution $HgCl_2$ to 1 part of formaldehyde), Zenker-formol, Heidenhain's SUSA sulfosalicylic acid, Romeis' fluid, or phosphotungstic acid (see Romeis, 1948) was used. After fixation, the pituitaries were embedded in paraffin in the routine manner and serial sections were cut at 2μ in the horizontal plane. For comparisons of preparations stained in various ways, series of sections were taken. Frequently, sections 2 and 5 of a series of six were stained by the sulfhydryl and disulfide methods of Barrnett and Seligman (1954a) and the adjacent sections were stained variously by the PAS-orange G technique of Pearse (1950), PAS or orange G alone, the aldehyde-fuchsin method of Halmi (1952) or the respective trichrome mixtures devised by Dickie (1944) and Goldberg and Chaikoff (1952). Occasionally, instead of a series, pairs of sections were stained. By adopting these procedures, it is possible to identify individual cells in adjacent sections stained by different techniques. Individual cells were identified by observing simultaneously similar fields of two preparations in two microscopes.

RESULTS

Since it was shown previously (Barrnett and Seligman, 1954a) that 80-TCA and Zenker's fluid were good fixatives for preserving sulhydryls and disulfides, these were employed in the present study. Alcohol-trichloracetic acid fixed anterior pituitaries of rats and mice showed few sulfhydryl groups (Fig. 1) and the cell types could not be distinguished with any degree of certainty. Sections of material fixed in 80-TCA and stained for both sulfhydryl and disulfide (Fig. 2) showed an increase in the over-all intensity of staining; although, cell types still were scarcely distinguishable. On the other hand,

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Explanation of Figures 1–4

Fig. 1. A 5μ paraffin section of the anterior pituitary of a male rat. Fixed in 1% trichloracetic acid in 80% ethyl alcohol (80-TCA), stained for sulfhydryl groups alone, photographed with green and yellow filters (Wratten B, no. 58; G, no. 15). There is a weak to moderate reaction in all cells, but the cell types cannot be distinguished. ×375.

Fig. 2. A 5μ paraffin section of the anterior pituitary of a male rat. Fixed in 80-TCA, stained for both sulfhydryl and disulfide groups, photographed as in Figure 1. The over-all intensity of the staining reaction in some cells appears to be enhanced; yet, cell types still cannot be distinguished. ×375.

Fig. 3. A 2μ paraffin section of the anterior pituitary of a male mouse. Fixed in Zenker's fluid, stained with orange G and a light nuclear counterstain of hematoxylin, photographed with a blue-green filter (Wratten H, no. 45). Typical "acidophiles" (a) that stain with orange G and which appear in varying shades of medium to dark grey in the photograph are shown by arrows. Also indicated are a basophile (b) and a chromophobe (c). ×500.

Fig. 4. An adjacent 2μ section to Figure 3. Fixed in Zenker's fluid, stained for sulfhydryl and/or disulfide groups, photographed with a green filter (Wratten B, no. 58). The "acidophiles" (a) that stained positively with orange G in Figure 3 react intensely for sulfhydryl and/or disulfide groups. The basophile (b) reacts weakly and the chromophobe (c) shows little or no reactivity for sulfhydryl and/or disulfide groups. ×500.

after fixation in Zenker's fluid followed by staining with sulfhydryl and/or disulfide methods, certain cells were intensely reactive (Fig. 4).

Figures 3 and 4 illustrate adjacent sections of a mouse pituitary, one of which is stained with orange G (Fig. 3) and the other for sulfhydryl and/or disulfide groups (Fig. 4).

It is apparent that the same cells are stained by both methods. The arrows in the photographs indicate those cells whose cytoplasm occurs about equally in each section. The cells that stained with PAS or aldehyde-fuchsin contained few sulfhydryl and/or disulfide groups (Fig. 4). Similar results were obtained with sections of pituitary of the rat, cat, dog and monkey.

Sections of rat or mouse pituitary stained for sulfhydryl and/or disulfide groups were compared with adjacent sections stained by the trichrome methods of Dickie (1944) and Goldberg and Chaikoff (1952). These methods as well as PAS-orange G staining revealed that the cells which are intensely sulfhydryl and/or disulfide positive are identical with the so-called "acidophile" cells. Chromophobes contained few or no sulfhydryl and/or disulfide groups (Fig. 4).

The specificity of the histochemical staining for sulhydryl or for the combination of sulfhydryl and disulfide was proven for the pituitaries by the methods reported elsewhere (Barrnett, 1953a; Barrnett and Seligman, 1954a).

DISCUSSION

The presence of compounds, presumably protein, which contain sulfhydryl or disulfide groups is dependent upon the fixative used. Although alcohol insoluble proteins containing sulfhydryl and disulfide groups may be demonstrated in the anterior pituitary after 80-TCA fixation, the quantity of stainable material is small and precludes identification of cell types (Figs. 1 and 2). When fixatives which are better protein precipitants are used, it becomes possible to distinguish a sulfhydryl and/or disulfide-containing cell type by the intensity of the reaction. Pituitaries may be fixed in a variety of fixatives mentioned earlier, but with the exception of Zenker's fluid, they compromise either the intensity of the staining reaction or the morphology of the pituitary cells.

It should be pointed out that the material which is preserved after Zenker's fixative, but not after 80-TCA fixative, is a protein which may contain sulfhydryls, disulfides or both. The sulfhydryls of a protein fixed in Zenker's, Zenker-formol and sublimate-formol fluids would be either oxidized to disulfide by the potassium dichromate or blocked by the heavy metal, mercaptide-forming agent (Hg^{++}). In the method for demonstrating both sulfhydryls and disulfides, under the conditions of reduction used (thioglycolate at pH 8.5), the sulfhydryls which are oxidized to disulfides by the dichromate would be reduced to sulfhydryl. The sulfhydryls blocked by the heavy metal (Hg^{++}) would be revealed again when the metal is removed by combination with the reducing agent. In addition, it should be borne in mind that the substances revealed after these fixatives could be disulfide-containing proteins that were soluble in 80-TCA but not in Zenker's fluid.

Although formalin may combine with sulfhydryls or disulfides (French and Edsell, 1945) to render them unreactive, under the conditions employed by Barrnett and Seligman (1954a), quite satisfactory staining resulted when comparison was made with other fixatives.

Our results show that after Zenker's fluid and certain other fixatives are used, the "acidophiles" are intensely reactive for sulfhydryl and/or disulfide. We are hesitant in calling this cell an "acidophile," but we do so because the cell which was reactive, was

also the cell which has been generally classified as an "acidophile" on the basis of its selective staining with the trichrome methods.

Histochemical techniques may be used for the identification of hormones or their precursors if four experimental criteria are satisfied. In the first place, the hormone or its precursor must contain a group which can be identified histochemically. Secondly, the histochemical method employed must be specific and the site of the reaction must be at the same locus as the group which is reactive. Thirdly, the histochemical method must be proven to react with the groups of the protein *in vitro* and in exactly the same manner as these groups are demonstrated in the tissue sections. Lastly, physiological experiments must be conducted which will reveal alteration in the staining reaction in a predictable manner as the hormone is stored or released. Such criteria have been met in the demonstration of disulfide groups in the anti-diuretic hormone (Barrnett, 1954) and insulin (Barrnett and Seligman, 1954b), as well as for certain enzymes (Barrnett, 1953b).

In essence, the observations reported here for the anterior pituitary satisfy the first two criteria, should the substance being demonstrated be a protein hormone(s) or its precursor(s). It is reasonable to expect to find disulfides in pituitary cells since it has been reported that growth hormone, lactogenic hormone, ACTH and LH contain disulfide groups (Li and Evans, 1948). It is also reasonable to suspect that any one or all of these hormones may be produced by "acidophiles." Experiments to satisfy criteria three and four, as presented above, will be reported in another communication.

SUMMARY

The histochemical technique of Barrnett and Seligman for the demonstration of protein-bound sulfhydryl and disulfide groups has been applied to sections of the anterior pituitary of various species. It was found that the "acidophiles" contained a significant amount of sulfhydryl and/or disulfide groups whereas basophiles and chromophobes contained little or no reactive material. The significance of this reaction is discussed.

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THE 1954 ANNUAL MEETING

The Thirty-sixth Annual Meeting of The Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, Thursday, Friday, and Saturday, June 17–18–19, 1954.

Dr. Roberto F. Escamilla, 655 Sutter Street, San Francisco, California, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Sir Francis Drake Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 19, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

Hotel reservations can be made through the San Francisco Convention and Visitors' Bureau, Room #300, Civic Auditorium, San Francisco, California. They should be accompanied by the official AMA application form which appears during December of 1953 in JAMA.

Arrangements have been made for members of this society who do not belong to the AMA to be assigned rooms during the days of the meeting of the Endocrine Society. Applications from non-members of the AMA should specify dates of arrival and departure and be accompanied by a deposit of $10 per room.

1954 AWARD AND FELLOWSHIPS

The award and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts, in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

The Ayerst, McKenna and Harrison Fellowship

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949. The 1950 Fellowship was awarded to Dr. Lawrence E. Shulman and in 1952 to Dr. John C. Laidlaw. The Fellowship will be in the amount of $5,000 and will be given on alternate years.

The Schering Fellowship in Endocrinology

This Fellowship will not be awarded in 1954 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1955.

AWARD

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishment of an investigator, not more than thirty-five years of age, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman; in 1953 the Award was presented to Drs. Sidney Roberts and Clara Szego. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.